ANALYTICAL STUDIES ON THE BENTIROMIDE TEST FOR PANCREATIC FUNCTION

By

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The author dedicates this dissertation to his wife, Susie, and their son, Jason, who have sacrificed much family time for this work to be completed. Without their patience, understanding and support this research would not have been possible.

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The urinary analysis of p-aminobenzoic acid which has been enzymatically cleaved from orally administered N-benzoyl-L-tyrosyl-p-aminobenzoic acid (bentiromide) has been proposed as a screening test for pancreatic function. The test as it is currently carried out has certain drawbacks that are due to poor selectivity of the analytical method and false positive tests encountered with disease states other than pancreatic dysfunction. The phosphorescence of p-aminobenzoic acid at room temperature under various analytical conditions was studied in an effort to develop an analytical method that is superior to those in current use for evaluation of the bentiromide test. Room temperature phosphorimetry of p-aminobenzoic acid was then applied to the analysis of urine samples from subjects undergoing the bentiromide test and the procedure was found to be sufficiently accurate and precise for clinical evaluation and was more selective than colorimetric methods.

An ion-pair high performance liquid chromatography procedure was also optimized for the analysis of p-aminobenzoic acid and its metabolites in urine so that metabolite concentration patterns could be correlated with disease states. It was found that patients with liver disease excreted significantly higher levels of the acetylated metabolite relative to the glycinated metabolite and this could be used to differentiate false positive bentiromide test results due to liver dysfunction. A room temperature phosphorimetric procedure for acetylated and glycinated metabolites of p-aminobenzoic acid was also developed but was shown to be of less diagnostic value than the chromatographic method.

This study demonstrates the applicability of room temperature phosphorimetry and high performance liquid chromatography to enhancement of the selectivity of the bentiromide test. The results presented here also provide a better understanding of p-aminobenzoic acid metabolism and the effects of metabolic conjugation on its phosphorescence characteristics.

CHAPTER I
INTRODUCTION

Orally administered bentiromide has been extensively studied as an indirect test of pancreatic function. Although the bentiromide test offers some advantages over other techniques, the method is relatively nonspecific with regard to the identification of pancreatic insufficiency. The work detailed here explores various analytical techniques in an effort to eliminate nonspecificity from both analytical and physiological sources. The analytical and clinical techniques employed in this work have been quite recently developed. Therefore, it is necessary to begin this dissertation with an overview of the bentiromide test and a discussion of pertinent theoretical concepts related to the analytical methods involved.

The Bentiromide Test of Pancreatic Function

The normally functioning pancreas is an efficient provider of amylolytic, proteolytic and lipolytic enzymes necessary for the digestion of food. Pancreatic secretion is mediated first by the vagus nerve in response to the sight, smell or taste of food (1). Additionally, the gastric mucosa can stimulate pancreatic secretion by the stimulation of stretch receptors or by gastrin release. The intestinal phase which is the major contributor to pancreatic stimulation is mediated by the release of the two hormones secretin and cholecystokinin (2). Secretin stimulates the pancreatic secretion of water and electrolytes whereas cholecystokinin acts to release the pancreatic enzymes.

The major pancreatic enzymes are α-amylase, lipase, chymotrypsin and trypsin.

Diseases of the pancreas are classified as inflammatory, traumatic, neoplastic or genetic. These diseases may vary widely as to the clinical picture and may present with or without exocrine pancreatic insufficiency (3). The general clinical features of pancreatic insufficiency are abdominal pain, diarrhea, and steatorrhea. Predisposing conditions such as cystic fibrosis or chronic alcoholism also provide valuable criteria for the identification of pancreatic disease. The diagnosis of pancreatic disease is difficult because of the anatomic location of the pancreas and a lack of adequate non-invasive diagnostic tests. The problem is compounded because certain clinical features and enzyme deficiencies may not be detected until 90% of the pancreas has been damaged (4).

Several tests of pancreatic function have been developed and can basically be categorized as direct or indirect tests. Direct tests of pancreatic function involve quantitative measurement of certain components of pancreatic secretions. These include the secretin and the secretin-cholecystokinin tests, both of which involve intravenous injection of pancreatic stimulants while monitoring the secretory response through aspiration of the duodenal fluid. Hormonal pancreatic stimulants vary greatly with respect to potency and the standardization of maximal normal secretory response has been difficult (5). Additionally, aspiration of duodenal fluid requires accurate placement of a nasogastric tube, usually by fluoroscopy, and results in radiation exposure (3). These procedures require hospitalization and some risk due to the invasive nature of such a technique. Although direct

pancreatic tests are considered the most reliable tests of pancreatic function, the disadvantages are obvious.

It is desirable to screen patients on an outpatient basis and use the direct tests as confirmation only in selected cases. Relatively noninvasive screening is the goal in development of indirect tests of pancreatic function. Measurement of pancreatic enzymes in body fluids (blood and urine) can be used as an indicator of acute pancreatic damage, but is not a measure of pancreatic function and is of little value in the diagnosis of chronic pancreatic disease (6). The Lundh test (7) is considered a reliable indirect test of pancreatic exocrine function although it involves duodenal intubation. The test is based upon chymotryptic activity on the substrate N-1-benzoyl-1-arginine-ethyl ester after a controlled test meal has been given. The enzymatic activity measured is an indirect quantitation of the amount of endogenous cholecystokinin released in response to the test meal. The test does not require intravenous administration of hormone stimulators and provides a functional evaluation. False positive results have been noted in the presence of other diseases (8) and the required enzyme analysis is analytically difficult to deal with.

The bentiromide test (9) is a relatively new approach to the indirect evaluation of pancreatic function. The test involves oral administration of the chymotrypsin-labile synthetic peptide N-benzoyl-l-tyrosyl-p-amino benzoic acid (bentiromide) shown in Figure 1-1. Digestion of this peptide by pancreatic chymotrypsin releases p-aminobenzoic acid (PABA) which can be subsequently absorbed, metabolized and excreted into the urine (9). The amount of PABA and its metabolites excreted in the urine, therefore, is an indirect measure of chymotrypsin

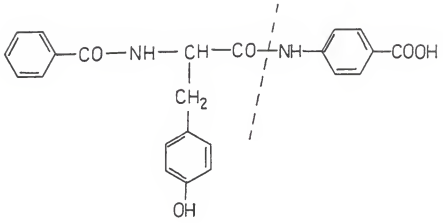


Fig. 1-1. Structure of bentiromide showing the point of cleavage by chymotrypsin (----------).

activity and thus an indicator of exocrine pancreatic function. Preliminary studies by Imondi and co-workers (10) suggested the test was a reliable indicator of pancreatic deficiency. They have subsequently shown that surgically induced pancreatic impairment can be accurately detected in a number of mammalian species (9). Since that time, the bentiromide test has been shown to be useful in determining pancreatic insufficiency induced by protein deficiency (11) and a number of clinical studies were carried out on human subjects (12-18). Bentiromide had been marketed for humans in Japan as early as 1980; however, the drug has just recently been approved for use in the United States. The peptide (bentiromide) was chosen on the basis of its sensitivity to hydrolysis by chymotrypsin and a general lack of hydrolysis by other pancreatic enzymes that may be present in the small intestine (9). The use of PABA provided an excellent marker molecule because it can be easily incorporated into peptides and is relatively nontoxic. It is also readily absorbed by the small bowel, rapidly excreted in the urine, and can be easily assayed.

The bentiromide test is typically carried out by administration of a 500 mg oral dose of bentiromide and collection of urine for 6 hours. The urinary levels of PABA at different doses show proportional changes and suggest that there is little difference in test results between doses of 15 and 50 mg/kg (19). It is possible, however, that very high doses may cause a situation in which the amount of chymotrypsin becomes rate limiting (20). The standard dose is 500 mg and dose adjustment can be made at a level of 15 mg/kg for pediatric patients. The amount of PABA and its metabolites found in the urine is usually expressed as the percent of the dose recovered and a normal value is considered as

anything above 57% (19). This is based on a study of 61 normal subjects in which the mean recovery was found to be 71.7 ± 7.2% and the cutoff was chosen to be the mean minus 2 standard deviations. Based on this cutoff point for normal values, the diagnostic sensitivity is 72% and specificity was determined to be 94.9% when compared to a pancreatic diseased population. The defining concepts of "diagnostic" sensitivity and specificity are outlined in equations 1 and 2 below.

$$\text{Sensitivity} = \frac{\text{Positive Tests}}{\text{True Positives} + \text{False Negatives}} \quad (1)$$

$$\text{Specificity} = \frac{\text{Negative Tests}}{\text{True Negatives} + \text{False Positives}} \quad (2)$$

It is evident from the above relationships that sensitivity is a measure of persons with the disease that go undetected whereas specificity is a measure of those who do not have pancreatic insufficiency but whose tests are positive. Both criteria are very important because both the need to identify disease and the avoidance of subjecting normal individuals to more invasive tests must be considered.

The sensitivity and specificity of the bentiromide test as it presently exists is not as good as those common to direct tests. The advantages of the bentiromide test lie in the practical utility of the method for screening since intravenous injections are not required, duodenal intubation is obviated, and there is no exposure to radiation. The analysis of PABA and metabolites in urine also eliminates the need for measurement of enzymes or unstable substances required by other methods. The analytical technique most commonly used

is the Smith modification of the Bratton-Marshall method (21) which detects total urinary arylamine concentration.

Physiological Factors Affecting the Bentiromide Test

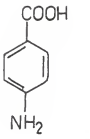
Although the bentiromide test seems quite attractive as a screening test for pancreatic function, there are several disadvantages that are not shared with other procedures. The test may not be useful in patients with severe hepatic disease since decreased metabolism could lower urinary PABA levels and thus create false positive test results (22,23). Additionally, patients with primary malabsorption in the small intestine may show false positive results due to decreased absorption of PABA (23). There has been some controversy over the degree of test specificity in patients with other diseases. With small bowel disease, patients may show symptoms similar to pancreatic insufficiency (24) that could cause difficulties with clinical diagnosis. This makes it especially important to be able to distinguish between the two disease states by diagnostic laboratory tests. Studies by Mitchell et al. (23) showed that 63% of the patients tested with small bowel disease had low bentiromide test results, although all of these patients had a normal direct test for pancreatic function. This incidence of false positive tests is totally unacceptable in light of the clinical picture.

Patients with small bowel disease and a normally functioning pancreas can be detected, however, with other diagnostic tests. An oral dose of xylose (25 g) followed by urinary analysis of that amount, which was absorbed through the small bowel and excreted, is an effective indicator of small bowel function and is not subject to chymotrypsin activity (25). This test has been administered concomitantly with the bentiromide test in dogs and good results have been obtained (26).

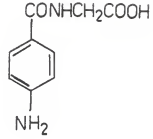
Although concomitant administration in humans has not yet been established as a reliable means of differentiation, separate administration of the two drugs has. Toskes and Greenberger (25) suggested a diagnostic algorithm for patients that present with symptoms typical of both small bowel and pancreatic disease. In this algorithm, patients with an abnormal bentiromide test and normal xylose test would be suspected of pancreatic insufficiency. Patients with abnormal results for both tests would have to undergo small bowel biopsy to assess small bowel function and patients with normal tests for both would be subjected to direct pancreatic function tests. With the use of this scheme, the authors proposed that the exact cause of symptoms could be determined in 85% of the cases.

For the case of liver disease, animal studies have shown bentiromide is metabolized to p-aminobenzoic acid (PABA) in the gut and subsequently conjugated by the liver to form both N-acetyl and glycine conjugates (Figure 1-2) which are the major metabolites found in urine (27,28). Glucuronide formation has also been observed for these derivatives (28), although this metabolic aspect may be poorly related to liver function since glucuronidase enzymes are present in many tissues (29).

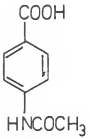
Studies have been carried out by Deiss and Cohen using PAHA synthesis as a model for liver function (30). In this work, various normal and pathological patients were given large doses (3 g) of PABA, and PAHA synthesis was monitored in serum samples. All pathological specimens tested demonstrated some negative deviation from the normal mean, although there was a great deal of variability depending on the type of liver disease. Studies have also shown that acetylation of PABA can be



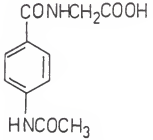
p-aminobenzoic acid (PABA)



p-aminohippuric acid (PAHA)



p-acetamidobenzoic acid (PAABA)



p-acetamidohippuric acid (PAAHA)

Fig. 1-2. Structures of p-aminobenzoic acid and its metabolites.

used as an indicator of liver function (31). There are, however, conflicting reports and many factors affect acetylation of PABA in vivo (32).

These studies show that PABA conjunction is decreased in patients with liver dysfunction and is dependent on the severity of the disease. It also follows that impaired conjugation may cause decreased urinary recovery of PABA metabolites resulting in false positive bentiromide test results. This was shown to be true in clinical studies involving liver patients and normal controls (22,23) where 50% of the liver patients studied had false low bentiromide test results. It also seems that conjugation impairment is dose related since the effect is more pronounced with high doses (1-2 g) of bentiromide. It appeared that PABA conjugation was impaired only in cases of severe, obvious liver disease and there are many commonly used laboratory tests to identify these patients. However, it would be advantageous to normalize the bentiromide test so that it could be used in patients with severe liver disease.

These physiologic interferences with the bentiromide test have been adequately compensated for by the determination of a PABA excretion index (22). In this approach, an equivalent test dose of underivatized PABA is given several days prior to bentiromide administration. The urinary recovery from pure PABA is then compared with that of bentiromide through a ratio of the two results. This has been shown to correct for both liver and small bowel disease but has the disadvantage of requiring two tests be carried out at different times.

Measurement of PABA metabolites in plasma samples after bentiromide administration has also been shown to detect pancreatic insufficiency

(33). This may also provide a means to avoid false positives due to liver dysfunction if unconjugated PABA alone were measured. Clinical studies involving liver patients, however, have not been done.

False negative bentiromide test results may also arise from physiological factors (24). In a study by Toskes (19), 20% of patients with well defined pancreatic disease showed normal test results. The explanation of this is not clear although it has been suggested that variations in duodenal pH may have been responsible (34). Other studies, however, have failed to confirm these results (35). Other factors that may contribute to the observed false negative results include carryover effects from pancreatic enzyme therapy (36), non-pancreatic enzymes that can cleave bentiromide (37), and bacterial overgrowth in the small intestine (38).

Analytical Factors Affecting the Bentiromide Test

False negative results can also be obtained due to the lack of specificity of the analytical procedure (39). Concurrent use of certain drugs (see Appendix I) and even some food substances (40) can cause a false elevation of urinary arylamine concentrations and possibly a false negative test. The drugs listed in Appendix I must be discontinued three days prior to the administration of bentiromide and could result in significant interruptions in necessary drug therapy. It is also difficult to totally regulate a patient's intake and some substances which are not suspect may also raise urinary arylamine levels.

Analysis of total PABA metabolites in urine from patients undergoing the bentiromide test have classically been carried out by colorimetric methods. The most commonly used method is the diazo-coupling method of Bratton and Marshall which was first used for

analysis of sulfanilamide (41) and subsequently modified by Smith (21). In this method, urine samples are hydrolyzed in 1.3 M HCl to convert PABA metabolites to the parent compound. The hydrolysate is then diluted according to the urinary volume collected over a 0-6 hr interval so that sample concentrations are within the range of the standard curve. The diluted hydrolysate mixtures which contain liberated PABA are then reacted with sodium nitrite via its primary amine group to form the diazonium chloride. Ammonium sulfamate is added to scavenge excess sodium nitrite so that it will not react in the next step. Finally, N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDA) is added and reacts with the diazonium chloride to form the azo-dye chromophore. Absorbance is measured at 550 nm. A more convenient colorimetric procedure has been developed by Yamato and Kinoshita (42) which uses the chromogenic reagent p-dimethylaminocinnamaldehyde (DACA). This method has the advantages of a one step color reaction, more stable reagents and a more stable colored endproduct. Although this method is slightly more convenient, it is no more specific than the Bratton-Marshall procedure because both reagents react with all primary arylamines present in urine hydrolysates. The non-specificity of the colorimetric methods was demonstrated in a study where 2 of 59 fasting subjects excreted significant aromatic amines in their urine.

Another possible source of error with these methods may arise from the acid hydrolysis procedure (43). Shosoki et al. (39) has shown that acid hydrolysis does not completely convert PAAHA (a major PABA metabolite) to the parent compound. Fortunately, PAAHA is primarily converted to PAHA which is a primary aromatic amine and therefore reacts to form a chromophore similar to that of the PABA-chromogen complex.

Differences in the absorptivities of these complexes and varible PABA metabolism could, however, lead to erratic results. The study by Shosoki et al. confirmed that alkaline hydrolysis completely converted all PABA metabolites and provides a better alternative since it is advantageous to measure a specific analyte rather than the additive contribution of two analytes.

High performance liquid chromatography (HPLC) with electrochemical detection has been proposed as a more specific alternative to colorimetric procedures (39). In this method, alkaline hydrolysis was used and the results indicated good precision and accuracy. The method also compared well with the DACA colorimetric procedure. The selectivity of the method was reported to be superior to colorimetric methods although this was not thoroughly documented with drug interference studies. The HPLC method is potentially superior analytically although the procedure is more costly and time consuming than colorimetric analysis.

Both physiological and analytical problems with the bentiromide test as it is presently carried out have been discussed in this section. The following sections will be dedicated to the establishment of a theoretical and practical treatment of the analytical methods involved in this research. Room temperature phosphorimetry will be used as an alternative to existing analytical methods in an effort to improve the selectivity of the test. In addition, concentration patterns of PABA metabolites will be studied by ion-pair HPLC so that physiological problems may be dealt with in this manner.

Room Temperature Phosphorimetry--Physical Aspects

The radiative transition from the lowest excited triplet state to the ground singlet state in organic molecules is called

phosphorescence. The triplet state is not normally accessible by direct excitation because electronic transitions from the ground singlet state to the triplet state are spin forbidden and occur with very low probability (44). The sequence of events that result in population of the triplet state is shown in Figure 1-3. If the absorption process (labeled A in Figure 1-3) is assumed to be a transition from the ground singlet state (S_0) to an excited vibrational level of an excited singlet state (S_1,S_2), the molecule can lose the absorbed energy through several pathways. These pathways are governed by the kinetics of the competing processes. The vibrational energy is usually lost by transferring energy to neighboring molecules through vibrational relaxation (VR) to the lowest vibrational level of the electronic state in which it resides. The competing process, however, in which an excited vibrational level loses its energy by emitting a photon equal in energy to the difference between its existing state and the ground singlet state, occurs at a much slower rate and thus with a much lower probability. Once in its lowest vibrational level, the kinetically favored event is usually internal conversion (IC) to the lowest excited singlet state (S_1). The molecule can then return to the ground state through internal conversion although this process is less probable than internal conversion from S_2 to S_1 because of the greater energy separation between S_1 and S_0. The radiative loss of energy from an excited singlet to the ground state is called fluorescence (F) and the efficiency with which it competes with internal conversion to the ground state depends on the type of molecule and its environment.

An alternate pathway to internal conversion and fluorescence is intersystem crossing (ISC) in which an electron changes the direction of

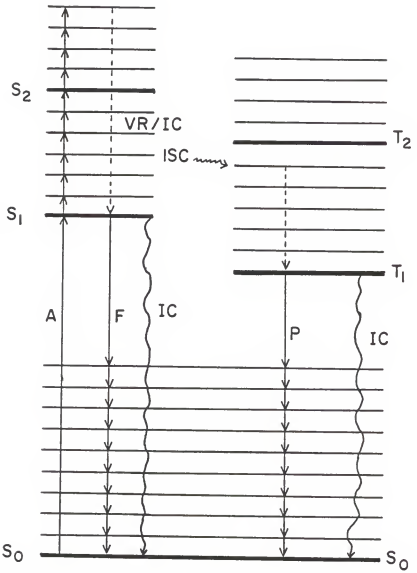


Fig. 1-3. Simplified energy level diagram showing singlet (S) and triplet (T) states for an organic molecule. Solid vertical lines represent absorption (A), fluorescence (F) and phosphorescence (P). Dotted lines indicate vibrational relaxation (VR). Internal conversion (IC) and intersystem crossing (ISC) are represented by vertical and diagonal wavy lines, respectively.

its spin within the molecule to place it in an excited vibrational level of the triplet state (T_1). Following intersystem crossing, vibrational relaxation to the lowest level of T_1 is very rapid. If the transition from the triplet state to the ground state is radiative, the process is called phosphorescence (P) whereas non-radiative transition again involves intersystem crossing.

Both fluorescence and phosphorescence are analytically useful processes although phosphorescence can only be observed from a limited number of molecules. Phosphorescence, like fluorescence, is most likely to occur in aromatic molecules and their derivatives which have restricted vibrational freedom.

Phosphorescence is generally distinguished from fluorescence on the basis of the longer decay-time of the former. Fluorescence decay-times are on the order of 10^{-9} to 10^{-7} seconds, whereas phosphorescence decay-times are generally between 10^{-6} and 10 seconds (45). The spin forbidden nature of phosphorescence is responsible for the relatively long decay-times observed (46). Phosphorescence emission maxima also occur at lower energy than corresponding fluorescence maxima (47). Intersystem crossing occurs in molecules where the lowest excited triplet state lies slightly lower in energy than the lowest excited singlet state (48).

The primary difficulty associated with analytical phosphorescence is that the triplet state, from which phosphorescence originates, is susceptible to radiationless decay by molecular collisions (44,49). This has led to the conduct of phosphorescence at very low temperatures in rigid matrices, in order to avoid these collisions. For many years, cryogenic spectroscopy was the only way to observe strong

phosphorescence from a variety of organic compounds (50-52). It has been shown more recently, however, that strong phosphorescence can be detected from a broad range of compounds at room temperature when rigidly adsorbed on a suitable support medium (44). Phosphorescence at room temperature has also been observed in solution when the emitting species are incorporated into stabilizing micelles which hinder the non-radiative deactivation pathways (53). These developments have opened up many new areas of interest in the application of phosphorimetry to chemical problems. They also provide additional insight into the phosphorescence phenomenon.

Because of the long lifetime of the triplet state and the fact that collisional deactivation is very effective in bringing about radiationless decay in fluid media, phosphorescence is rarely observed in solution at room temperature. There have, however, been various attempts made to observe phosphorescence in solution at room temperature. In a limited number of cases, phosphorescence at room temperature has been observed from solutions that have been thoroughly degassed to remove small amounts of dissolved oxygen that quench the triplet state (54). Some compounds show phosphorescence in the gas phase due to a decrease in the frequency of molecular collisions at low pressures (55-58). A number of compounds exhibit strong phosphorescence at room temperature when embedded in rigid media such as glasses and plastics (59). In each of these cases, observed phosphorescence at room temperature has been either too weak for analytical purposes or there have been problems with sample preparation (60).

Substrate Interactions in Room Temperature Phosphorescence

Many polar and ionic organic compounds have been found to exhibit phosphorescence at room temperature when adsorbed on materials such as silica, paper, alumina or cellulose (61-64). Since these early reports, a large number of compounds capable of phosphorescence in this manner have been identified. The phosphorescence of organic compounds adsorbed on solid supports is called room temperature phosphorescence (RTP).

Studies by Schulman and Walling (64) showed that nonionic forms of compounds displayed very little RTP relative to their ionic counterparts. These studies suggested that surface adsorption held the phosphors rigidly enough to limit collisional deactivation since adsorption of an ionic compound should be considerably stronger than that of the nonionic form. Subsequent studies have shown that reduction of the polarity of the adsorbant greatly decreases the intensity of RTP (64). It is now generally accepted that molecules capable of strong adsorption onto the support material prove to be the most efficient phosphors (44).

Hydrogen bonding of the phosphor to the support material was also investigated as a means to restrict collisional deactivation of the phosphorescent compound (65). These experiments showed that reduction of the number of hydrogen bonding sites on the adsorbant produced a decrease in RTP intensity. Further studies supported the involvement of hydrogen bonding by comparing the RTP intensities of ortho and para-aminobenzoic acid adsorbed on sodium acetate (66). Para-aminobenzoic acid, which is strongly hydrogen bonded to the support according to IR data, demonstrated very intense RTP whereas o-aminobenzoic acid did not show RTP. The ortho isomer did not reveal strong hydrogen bonding to

the support, probably due to intramolecular hydrogen bonding of the substituents. The support-phosphor interaction is not yet fully understood and a number of anomalies exist which suggest the involvement of factors in addition to those already discussed.

Influence of the Matrix on Room Temperature Phosphorescence

There are many environmental effects on RTP intensities and investigations of these effects have led to a better understanding of phosphor-support interactions. It was noted by some initial investigators that thorough drying of compounds adsorbed on paper and other supports was necessary to observe RTP. The effects of moisture on the RTP of sodium 4-biphenylcarboxylate was investigated by measuring the RTP intensity as a function of relative humidity (65). The emission intensity was found to decrease dramatically with increasing relative humidity. The reduction in RTP intensity was attributed to displacement of the hydrogen-bound phosphor by water, since hydrogen bonding was considered the primary adsorption process. Other studies showed that RTP could be observed without drying if anhydrous solvents were used and the sample was protected from humid air (67). These results support the adsorption hypothesis since competition for hydrogen bonding sites by water would increase the mobility of the phosphor and, hence, the chances of collisional deactivation.

Triplet state oxygen is a potent quenching agent of the excited triplet state through energy transfer. It is, therefore, surprising that strong RTP has been observed even under an atmosphere of pure oxygen. This suggests that the sample matrix somehow inhibits oxygen quenching (64). Subsequent studies have shown, however, that oxygen quenching does occur, to an extent depending on the sample matrix and

the phosphor (65,68). A possible explanation for this may be that some oxygen is trapped in the sample matrix when dried in the presence of oxygen since the more strongly the phosphor is adsorbed to the substrate, the greater the quenching effect of oxygen. Oxygen quenching of phosphorescence also appears to occur to a greater extent in humid atmospheres, which suggests that there is greater penetration of oxygen to the phosphor in the presence of water (67).

The addition of external heavy atoms to the phosphorescence matrix has been commonly known to result in a decrease in fluorescence yield with a corresponding increase in the phosphorescence yield (49). Heavy atoms are necessary to observe RTP from certain nonpolar polynuclear aromatic hydrocarbons (68). These compounds should still be quite susceptible to collisional deactivation since strong adsorption between a nonpolar compound and a polar adsorbant would be unexpected. One possible explanation of this phenomenon might be the formation of polar π-complexes between the compound and the heavy atom perturber that provide protection against collisional deactivation (68). The polar π-complexes may also interact more strongly with the substrate, which provides another possible mechanism for the increase in phosphorescence yield.

The heavy atom effect has been attributed to increased spin-orbit coupling induced by the heavy atom (69-74). This makes population of the triplet state more likely through an increase in the singlet-triplet intersystem crossing rate and results in a decreased fluorescence yield and an increased phosphorescence yield. This also accounts for the observation that phosphorescence lifetimes are generally shortened in the presence of heavy atoms (44). Although radiative decay is usually

more strongly enhanced, the opposite effect has also been observed (75). Sodium iodide has been successfully used to enhance RTP intensities either by preparing samples in iodide solutions or by spotting the solutions onto samples prior to drying. The increases in RTP intenstities for some compounds upon addition of sodium iodide were reported to be much too large to be explained totally by an increased intersystem crossing rate (75). This supports the polar π-complex adsorption theory.

Spectral Characteristics

Spectral features observed in RTP are generally similar to those found at low temperatures (76). Room temperature phosphorescence spectra generally demonstrate lower resolution of vibrational structure than low temperature phosphorescence, due to increased vibrational freedom at higher temperatures (77). Red shifts, typically less than 10 nm, are also observed in room temperature spectra when compared to corresponding low temperature spectra. E-type delayed fluorescence is more readily observed at room temperature than at very low temperatures due to the lack of thermal energy to excite the triplet molecule back to the lowest excited singlet state at low temperatures (46). Only minor effects on spectral features are observed for the same compound adsorbed on a variety of support media (14). The pH of sample solutions can have a dramatic effect on RTP spectral properties (64,78). Although this has not been investigated, it is reasonable to assume that similar effects may be observed when changing from acidic to basic support media. Although sample matrices have little effect on RTP spectral positions, lifetimes and intenstities can be significantly affected (79,80). In addition to the heavy atom effect, many other compounds such as

potassium hydroxide, boric acid, and sucrose have been found to affect either phosphorescence intensity or phosphorescence lifetime when added to the RTP matrix (44).

Phosphorescence at Room Temperature in Solution

Phosphorescence observed at room temperature in micelle solutions, provides an interesting aspect for further study. As mentioned previously, limited phosphorescence in solution at room temperature has been observed but only after vigorous nitrogen purging to remove dissolved oxygen. Some compounds which do not exhibit phosphorescence upon nitrogen purging alone, show significant phosphorescence in purged solutions containing certain detergents above their critical micelle concentration (38). A possible explanation of this effect is micellar stabilization of the triplet state (50). It is proposed that phosphorescent molecules are shielded from collisional deactivating processes when they are within the protective environment of the micelle. These studies have been carried out in both aqueous and non-aqueous solutions (81,82).

Studies of phosphorescence from micellar solutions have usually employed internal or external heavy atoms. The replacement of sodium by a monovalent heavy metal ion (e.g., Tl^+) in sodium lauryl sulfate micellar solutions dramatically increased phosphorescence intensities reaching a maximum at 10-20% heavy metal ion replacement (82). No RTP was observed in aqueous micellar solutions of naphthalene, pyrene, or biphenyl in the absence of heavy atoms although sensitivities comparable to those attained in other phosphorescence methods were reported when the heavy metal was added. Analytical Figures of merit have been reported for a limited number of compounds in aqueous micellar solutions

and are reported to generally compare favorably to low temperature measurements (82).

Room Temperature Phosphorimetry--Application

There are several published sources of specific information on RTP spectral characteristics and analytical Figures of merit for a number of phosphorescent compounds on various support media (83,84,85). Data pertaining to compounds of interest in biochemistry, clinical chemistry, pharmaceutics and environmental chemistry have been reported. Limits of detection for these compounds generally range from subnanogram to submicrogram quantities (85). Room temperature phosphorescence provides much greater selectivity than other luminescence techniques due to the fact that fewer compounds demonstrate RTP than fluoresce or phosphoresce at room temperatures (84). Room temperature phosphorescence obviates cumbersome techniques necessary to observe phosphorescence at low temperatures, and the capability for automation makes RTP a favorable alternative to low temperature phosphorimetry.

Quantitative Applicability

Equations developed by Winefordner describe a linear relationship between RTP intensity and concentration that departs from linearity at high concentrations much like low temperature phosphorescence (83). This effect, in practice, may occur at lower concentrations than predicted due to inner filter effects, triplet-triplet annihilation and various other factors (45). The quantitative capability of RTP has been demonstrated for a number of compounds and shows promise for practial utility with real samples.

The choice of the proper compound and support for RTP is an important factor in the success of an analysis. Highly conjugated compounds with one or more aromatic rings are good candidates. The

compound should have a high phosphorescence quantum yield at low temperature and contain highly polar or ionic groups in order to have a high probability of showing RTP. As mentioned previously, certain nonpolar compounds exhibit RTP but only with the aid of a heavy atom perturber.

Useful support materials usually contain either hydroxyl groups or numerous ionic sites (83). The most common support material has been paper. Silica gel and sodium acetate have also been widely used as RTP support materials. Selection of an optimum support has been largely trial and error, up to this point, and investigation is still in the early stages.

Sample preparation in RTP usually involves application of the sample solution (3-5 μl) onto the support and thoroughly drying it. The drying step is especially critical due to the dramatic decrease in RTP intensities when the sample is exposed to moisture. Drying has been accomplished with heat lamps, blow dryers, dry nitrogen purge and laboratory ovens although some compounds may require maintenance under anhydrous atmospheres (83).

For quantitative purposes, sample application volumes should be precisely measured and the amount of surface area covered should be controlled (86). Drying times should be optimized and kept constant. The presence of oxygen and temperature fluctuations (87) have been implicated as sources of imprecision in RTP and these variables should be controlled by drying under nitrogen at constant temperatures for best results. The pH of the analyte solution and support should be optimized to provide maximum RTP intensities. Some heavy atom perturbers have been shown to preferentially enhance RTP emission of individual

components in a multi-component mixture. This observation has been used to selectively determine each of the components in a multi-component system and has been termed selective external heavy atom perturbation (SEHAP)(88).

Instrumentation for Room Temperature Phosphorimetry

Although there is no commercially available instrumentation specifically designed for RTP detection, there have been several modifications of conventional luminescence instruments to accomodate the technique. Typically, the sample is mounted on a holder which allows positioning of the paper or other support material so that optimum excitation-emission geometry can be attained. The holder takes the place of the quartz Dewar flask used in low temperature measurements and the sample compartment is usually equipped with a gas purging system to avoid quenching by oxygen and humid air. The unit may also be temperature controlled to avoid possible imprecision as discussed earlier. The Aminco-Bowman spectrophosphorimeter has been reported to be suitable to receive these modifications (76).

A modification of the Schoeffel spectrodensitometer which allowed viewing of RTP from several spots on one sheet of paper or thin layer chromatography plate was described by Ford and Hurtubise (89). The modified reflection assembly allowed the photomultiplier-source exit distance and photomultiplier angle to be varied to maximize reflected RTP reaching the detector system. These modifications were made to accommodate a rotating disc phosphoroscope.

The most innovative instrumental design was constructed to demonstrate the automation capability of RTP (90). This system employed a filter paper guide that allowed continuous feeding of a paper tape

under a syringe for sample spotting, through a drying oven and finally into a sample compartment of a modified spectrophosphorimeter. Emission from the analyte spot was integrated as the paper tape moved at a continuous speed over the aperture. The semiautomated system was capable of measuring 7 to 8 samples per minute.

Several other instrumental techniques have been described as means to improve RTP analysis. Ellipsoidal and parabolic mirrors have been employed to increase the collection efficiency of the luminescence from small volume samples which are typical of RTP (91). Synchronous scanning of excitation and emission monochromators (92) and derivative techniques (93) have both been used as means to improve the selectivity of the method.

Advantages of Room Temperature Phosphorescence

Sensitivity, selectivity, automation capability and avoidance of cryogenic techniques are all significant advantages of RTP analysis. Other advantages of RTP that warrant consideration are the small amounts of sample required and the possibility of direct spectral identification/quantification of separated analyte spots on paper and thin layer chromatographic plates. The linear dynamic range of RTP analytical curves and measurement precision has been reported as favorable in some instances (84).

In contrast, there are also a number of limitations that are observed in RTP methods. The uniformity in thickness and porosity of available supports varies considerably from lot to lot causing problems with accuracy and precision. RTP is also more susceptible than low temperature procedures to scatter and background errors associated with solid surface instrumental measurements (85). Both of these limitations

could be minimized by production of substrates which are more suitable for RTP measurement and development of better solid surface instrumental designs.

A major limitation of RTP as an analytical method lies in the phosphorescence background observed between 400 and 600 nm (94). Virtually every support that has been evaluated and shown to produce significant RTP signals also exhibits considerable background interference (84). Various pretreatments, experimental conditions, and instrumental adaptations have been examined as a means to lower this background with little success. Heavy atom perturbers must also be used with caution since they may have a similar enhancement effect on the background signal. Even with these high background levels, limits of detection for strongly phosphorescent compounds can be in the subnanogram range and the consequence of the background is that appropriate corrections for the blank must be made.

Phosphorescence at room temperature arising from micellar stabilization might seem to be a viable alternative although studies are quite recent and have not been widely documented. The vast number of micellar systems available could provide excellent possibilities with respect to analytical applications. These possible advantages to the micellar stabilization technique, however, are overshadowed by the procedural difficulties. The very strict degassing requirements, concentration dependence, and susceptibility to impurities of the system create limitations to the analytical usefulness of the method.

Quantitative analysis by RTP is a very sensitive and selective method for a number of biologically important compounds. Application of RTP to clinical analysis has hardly begun, probably due to a lack of

familiarity with the technique. The potential for both quantitative and qualitative analysis has been established, however, and RTP methods should find their way into practical analytical situations in the near future.

High Performance Liquid Chromatography--Theory

Chromatography is essentially a physical method of separation in which components are distributed between two phases, a stationary bed and a mobile liquid or gas flowing through this bed. Liquid chromatography refers to any chromatographic process in which the moving phase is a liquid. Open-bed liquid chromatography has been used routinely as a separation method for the past 40 years although original reports date back to 1903. The growth of liquid chromatography has occurred in periodic spurts corresponding to major innovations in techniques and instrumentation.

Liquid chromatography can be divided into several subgroups based on the nature of the stationary phase and the separation process. In adsorption chromatography, the stationary bed is a solid on which separation is achieved through adsorption-desorption steps. Partition chromatography involves separation based on distribution between the liquid stationary phase and the mobile phase. Chromatographic separations based on ion-exchange principles have also been developed in which the species to be separated are charged and are retained by the oppositely charged stationary phase according to their relative charge densities. Separations based on molecular size are carried out with stationary phases having precisely controlled pore sizes. In these methods, the sample is simply sieved according to molecular size differences as it is washed through the stationary phase. Further

subdivisions of liquid chromatography exist although the main divisions are simply those of adsorption versus partition methods. In all types of chromatography, smaller stationary phase particles provide the most efficient separations due to increased total surface area. Smaller particle size, however, creates practical problems since the mobile phase moves very slowly through a dense particle bed. This has led to the conduct of liquid chromatographic separations at high pressures for the purpose of lowering analysis time while maintaining efficient separation. The method of applying high pressures to liquid chromatographic columns in a closed system has since been termed high performance liquid chromatography and has brought about a new era in chromatographic analysis. The following section describes some of the theory involved along with practical considerations pertinent to this dissertation.

Migration and Retention

The differential migration of compounds which serves as the basis for chromatographic separations is governed by equilibrium concepts. Due to the nature of the flowing system, however, it will be shown that complete equilibrium is valid only at the center of the migration zone of the compound of interest. If chromatographic migration is to be related to the equilibrium distribution of molecules between phases, we must refer to the center of the zone and have a rapid exchange of molecules between phases.

Chromatographic separation of components in a mixture is achieved through differences in their equilibrium distribution (K) between two phases. If C_s and C_m are the concentrations of a component in the stationary and mobile phases, respectively, then,

$$K = C_s / C_m \qquad (3).$$

Migration is assumed to occur only when molecules reside in the mobile phase and each time a molecule affixes itself to the stationary phase its migration is interrupted. A zone of component molecules is then observed to migrate smoothly at some fraction (R) of the mobile phase velocity. Since migration can be viewed as equivalent to the probability of molecules existing in the mobile phase, the fraction of molecules in the mobile phase at equilibrium is equal to the same parameter R and denotes complete equilibrium. However, R (known as the retardation factor) is an average time fraction and zone spreading occurs as a result of fluctuations from this value. Therefore, R is applicable only at the zone center and molecules at the front and rear of the zone deviate from true equilibrium. The value R, having been established as equal to the equilibrium fraction of solute in the mobile phase, can be related to the equilibrium solute fraction (1-R) in the stationary phase. Thus, the term R/(1-R) is the ratio of the mass or number of moles of solute in the mobile phase to that in the stationary phase. This then is equal to the product of the solute concentration in the mobile phase and the volume of the mobile phase ($C_m V_m$) divided by the corresponding term ($C_s V_s$) for the stationary phase and

$$\frac{R}{(1-R)} = \frac{C_m V_m}{C_s V_s} \qquad (4).$$

If the partition coefficient (K) is substituted from equation (3), we have

$$\frac{R}{(1-R)} = \frac{V_m}{KV_s} \qquad (5).$$

If equation (5) is solved for R, an expression equivalent to the classic equation of Martin and Synge (95) is produced:

$$R = \frac{V_m}{V_m + KV_s} \qquad (6).$$

Equation (6) has been derived as though there were a volume associated with the stationary phase, as is the case for the various classes of partition chromatography. However, for adsorption chromatography, V_s can be replaced by the surface area of the stationary phase to attain an equivalent expression. If retention is due to both adsorption and partition mechanisms (e.g., partial adsorption in partition chromatography; Figure 1-4), equation (6) can be extended to the following (96):

$$R = \frac{V_m}{V_m + K_i V_{si}} \qquad (7).$$

where K_i and V_{si} are the equilibrium constant and apparent volume of stationary phase which are experimentally derived and account for the adsorption process. At any rate, R can always be related to the equilibrium partition coefficient and the amount of each phase. Various forms of this equation exist, many of which can be used in chromatographic practice to predict the degree of retention for a given system. A similar parameter (k'), which is essentially the reciprocal

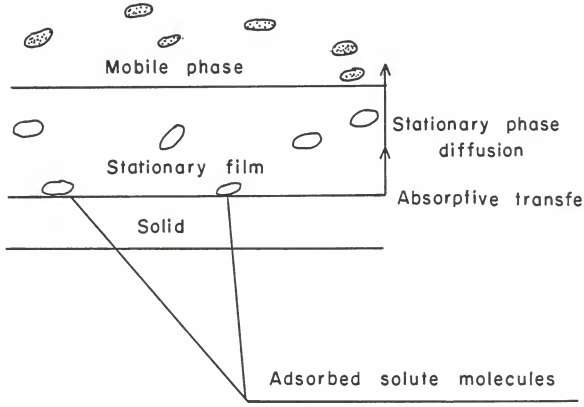


Fig. 1-4. Idealized system for simultaneous adsorption and partition processes in liquid chromatography showing two mass transfer terms.

of R, is popular among chromatographers and is referred to as the capacity factor (97).

Zone Spreading

The effectiveness of chromatographic separation depends on two important factors: control of the relative migration rates and the amount of zone spreading. These two factors are equally important in attaining a desired separation since the separation is equally enhanced by either doubling the difference between the migration rates of different solutes or halving the spreading of peaks. The control of migration rates is accomplished by employing specific interactions (e.g., choice of the stationary and mobile phases) and is beyond the scope of this treatment. Therefore, only zone spreading will be considered here. The physical processes that give rise to zone spreading are present in all forms of chromatography and are of particular importance when dealing with nonlinear partition isotherms (Figure 1-5; 98). Exact linearity of partition isotherms is rarely attained in liquid chromatography and ideal conditions would result in each zone maintaining its original shape as it migrated through the column.

There are three main contributions to zone spreading: eddy diffusion, longitudinal diffusion, and mass transfer. Eddy diffusion arises from the differences in mobile phase velocity attributed to different flow paths that solute molecules take through the particle bed and spreading is caused by faster movement of solute molecules thorugh wider paths relative to those in narrow paths. Longitudinal diffusion is the band broadening process that results from diffusion of solute molecules in the direction of mobile phase flow and becomes significant

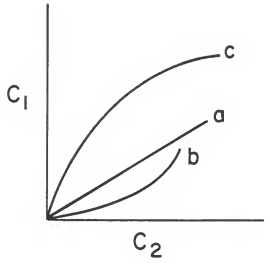


Fig. 1-5. Three types of partition isotherms (C_1 is the total concentration in phase one and C_2 is the total concentration in phase two). Curve a represents the ideal situation where the distribution ratio does not vary with relative concentrations. Curve b shows variations which would be observed for solute association. Curve c demonstrates the isotherm encountered frequently when phase one is an adsorbed phase.

only under conditions of stopped-flow and at low flow rates. Mass transfer spreading of the chromatographic band is primarily due to differences in the residence time of solute molecules on the stationary phase. Molecules that diffuse deeper into the stationary phase or are adsorbed more stongly spend more time in the stationary phase before they return to the mobile phase and will thus lag behind the main concentration of solute molecules. The relative importance of each of these contributions to zone spreading varies with the type of chromatographic system and conditions and is due mainly to the differences between physical properties of mobile phases. Mass transfer effects are the most significant; therefore, diffusion processes contributing to zone spreading will simply be described whereas mass transfer equilibrium will be considered in detail.

The Theoretical Plate Concept. The theoretical plate concept is very useful in the description of zone spreading. This model was introduced into separation science to describe the distillation process. The efficiency of the distillation separation is determined by the number of condensation-volatilization steps required to attain a specific degree of purity. These hypothetical steps occurring during the repeated vapor-liquid equilibria were called theoretical plates. Understanding this process as it pertains to chromatography requires one to consider that the chromatographic column is divided into discrete segments corresponding to individual extraction tubes. Each segment contains an amount of mobile phase and stationary phase in which complete equilibration of solvent distribution has occurred. As a sample is added to the column, it enters the first such segment and solvent equilibration occurs. A fractional amount of solvent remains in

the mobile phase of the first segment to be passed on to the second and another mobile phase volume unit is added to the first stationary phase segment. As the process continues, a pattern of solute distribution develops which results in the observed separation. These segments are the theoretical plates and an entire chromatographic column contains a specified number (N) of these plates. A more detailed description of the theoretical plate theory is presented by Purnell (99).

The theoretical plate model has two functions. First, it serves to describe chromatographic processes in the well defined terms of countercurrent distribution and, second, it provides a parameter H (the height equivalent to a theoretical plate) to characterize zone spreading and resolution. The use of this model to describe chromatographic processes is only approximate because the assumption that equilibrium is reached throughout the zone is not valid (100). However, the use of the parameter (H) is acceptable as a descriptive term to evaluate column efficiency.

For given chromatographic conditions, the parameter H is approximately constant for different zones in the chromatogram and is, therefore, a measure of column efficiency. The parameter H is related to the number of theoretical plates (N) by the following expression:

$$H = L / N \qquad (8)$$

where L is the length of the column. Thus, H measures column efficiency per unit length and small values of H (large N values) correspond to more efficient columns. The parameter H can also be expressed directly in terms of zone spreading by

$$H = \sigma^2 / L \qquad (9).$$

The distance migrated by the zone center is represented by L and σ corresponds to the standard deviation of the Gaussian curve which approximates zone distribution. The standard deviation of the Gaussian curve is a direct measure of zone spreading equivalent to roughly the quarter-width of the zone at the baseline.

Having described zone spreading in terms of the theoretical plate height, we can now consider individual contributions to zone spreading in these terms.

The plate height contribution due to longitudinal molecular diffusion (H_L) is given by

$$H_L = 2 \gamma D_m / V \qquad (10)$$

where γ is an obstruction factor that accounts for hindrance of diffusion by the column packing. The diffusion coefficient of the mobile phase is represented by D_m and V is the mobile phase velocity.

Giddings (100) has shown that the contribution to H from eddy diffusion is

$$H_E = 2 \lambda d \qquad (11)$$

where d is the packing material particle diameter and λ is a packing constant.

Mass transfer and nonequilibrium. Mass transfer in the stationary phase contributes to zone spreading by two basically different mechanisms or by a combination of both. In adsorption chromatography, an abrupt molecular attachment or detachment process is the critical

step leading to sorption or desorption of solute molecules. Solvent molecules can detach from the stationary phase only if they possess sufficient energy to cause rearrangement or rupture of chemical or physical bonds. Partition chromatography, however, may be quite different in the mechanism of sorption-desorption in that penetration into and removal from the stationary phase are controlled by diffusion where a change occurs gradually. Mass transfer has been treated in terms of the random walk model (101,102) in which solute molecules are viewed as being displaced from the stationary phase in discrete forward or backward steps. The model is correct, however, only after a time period sufficient for solute molecules to form a Gaussian shaped zone profile has elapsed and should be reserved only to provide a basic understanding of the principal effects on zone spreading.

A more accurate way of viewing the chromatographic process is that of a continuous flowing system. As a moving concentration pulse (solute zone) flows through a given column region, the concentration will increase to a maximum and then decrease back to zero. In the leading edge of the zone, a finite time is required to bring the stationary phase concentration to equilibrium with the continuously increasing concentration in the mobile phase. As long as the concentration continues to increase in the mobile phase, complete equilibrium of the stationary phase remains just out of reach with its concentration lagging slightly behind the mobile phase concentration (103,104). The reverse of this process is true for the trailing end of the zone since a new influx of mobile phase brings with it continuously more dilute solutions. This results in the stationary phase concentration being slightly higher than the equilibrium concentration in the mobile phase

after the zone center has passed. A schematic representation of this equilibrium lag in the stationary phase can be seen in Figure 1-6. The degree of equilibrium lag is dependent on the rate of mass transfer and will be slight if the rate of sorptive-desorptive exchange is large or if the zone migrates very slowly. This degree of nonequilibrium is very important since it determines the extent of zone spreading due to mass transfer and largely determines column efficiency.

The nonequilibrium theory is based on changes in solute concentrations which result from the flow and kinetic processes observed in chromatography (105). Since the extent of departure from equilibrium is directly responsible for zone spreading, the concern is to quantify the effect in terms of equilibrium departure parameters:

$$\varepsilon_m = \frac{C_m - C_m^*}{C_m^*} \tag{12}$$

and

$$\varepsilon_s = \frac{C_s - C_s^*}{C_s^*} \tag{13}$$

where ε_m and ε_s are equilibrium departure terms for the mobile and stationary phases, respectively, and C_s and C_m denote concentrations per unit volume of packing material. The asterisk refers to equilibrium conditions at the zone center. From equations 12 and 13, it is evident that the ε term is the fractional departure from equilibrium.

The equilibrium departure term can be related to column efficiency through the plate height parameter with the following relationships. The change in solute flux (ΔJ) is proportional to the overall concentration gradient as the mobile phase moves through any cross

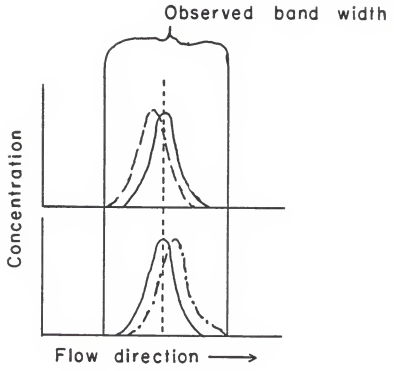


Fig. 1-6. Diagram showing the stationary phase concentration lag (upper diagram) and mobile phase concentration forward displacement relative to the equilibrium zone center for liquid chromatography.

sectional area at a mean mobile phase velocity (v) and can be taken as

$$\Delta J = C_m^* \varepsilon_m v \qquad (14).$$

The flux can also be represented in terms analogous to diffusion, where D is the apparent diffusion coefficient for zone spreading as in the equation

$$\Delta J = -D \, \partial c/\partial z \qquad (15).$$

The partial derivative $\partial c/\partial z$ represents the concentration gradient along the column axis (the partial form indicates that there is more than one variable and that all but one must be kept constant). Combining equations 14 and 15,

$$D = - \frac{C_m^* \varepsilon_m v}{\partial c/\partial z} \qquad (16).$$

The plate height contribution is related to equation 16 by

$$H = 2D/Rv \qquad (17).$$

Therefore, substituting for D and expressing C_m^* as RC, we have

$$H = \frac{-2(RC)\varepsilon_m}{R \, \partial c/\partial z} \qquad (18).$$

Simplification of equation 18 yields

$$H = \frac{-2\varepsilon_m}{\partial \ln c/\partial z} \qquad (19).$$

This simple expression achieves the purpose of relating plate height to the equilibrium departure term. However, this does not mean that H is inversely proportional to $\partial \ln c/\partial z$ or independent of v because the mobile

phase departure term will always be of a form capable of cancelling the $\partial \ln c/\partial z$ term and introducing a term for v (see the following expression):

$$\epsilon_m = \frac{-(1-R)v}{k_d + k_a} \frac{1}{C_m^*} \frac{\partial C_m^*}{\partial z} \qquad (20).$$

where k_a and k_d are the first order rate constants for adsorption and desorption onto the mobile phase. If it is assumed that in partition chromatography the liquid stationary phase is a uniform film of depth d, the diffusional mass transfer coefficient in the stationary phase (D_s) can be similarly related to H by means of nonequilibrium concepts. The equilibrium departure term for diffusion controlled mass transfer through a liquid stationary phase can be expressed in terms of the following parameters:

$$\epsilon_m = - \frac{Rv}{Ds} \frac{\ln c}{z} \frac{(1-R)d'^2}{3} \qquad (21).$$

Therefore, relation to plate height is simple since this parameter has already been defined for adsorptive processes in equation 19. A direct substitution of equation 21 into equation 19 with simplifications results in the relationship:

$$H = 2/3\ R(1-R)\ \frac{d'^2 v}{Ds} \qquad (22).$$

Equation 22 is important since it shows that the plate height parameter (and thus column efficiency) is proportional to flow velocity, the square of the packing material film depth and the inverse of the diffusion coefficient in the stationary phase. These parameters can be

adjusted in practice and increased column efficiency is easily affected by the minimization of d' and the choice of stationary phase liquids in which D_s is large. Flow velocity is not usually minimized to affect column efficiency because of the increased analysis time which results. Interestingly, equation 22 is the same as that derived for the partition case by random walk theory (101). The two values differ only by a numerical constant with the above expression being more correct. In practice, the numerical constant should be replaced by a variable term (usually q) which is called the configuration factor.

The value of q depends on the shape of the pool of partitioning liquid (106) and the value of 2/3 derived by nonequilibrium theory is characteristic of a uniform film.

Application of Liquid Chromatographic Theory

In the previous section, the basic theoretical concepts of migration and zone spreading were discussed. The plate height parameter concept was described in terms of theoretical plate theory and related to the nonequilibrium model. The known complexity of chromatographic packing materials has led to many extensions and generalizations of the simple nonequilibrium theories discussed here (107-109). For example, complex mass transfer treatments can account for nonuniformity of surfaces in adsorption chromatography. In addition, it should be obvious to a practiced chemist that Figure 1-4 should include mobile phase diffusion and interfacial transfer processes to be correct. If generalized nonequilibrium theory is applied, not only can experimental observations be interpreted in terms of theory, but specific information with regard to the degree of nonuniformity versus column efficiency can be obtained.

Although we have seen that mass transfer terms constitute only a part of the total plate height, they are by far the most important from a practical standpoint and are the most interesting from a theoretical point of view. Plate height contributions due to other factors such as longitudinal diffusion and eddy diffusion in the mobile phase have been treated less rigorously throughout this introduction. Also, skew of the chromatographic migration zone (usually due to cases of slow equilibrium) has not been discussed. The attempt has been to describe the basic theoretical groundwork from which more complex treatments can precisely characterize chromatographic phenomena.

Bonded-Phase Liquid Chromatography

In this section, the practical aspects of liquid-liquid chromatography will be addressed with emphasis on the bonded stationary phase packings relevant to this work. Bonded-phase chromatography (BPC) is the most widely used means for high performance liquid chromatographic separation (110). Bonded-phase chromatography column packings are those with organic stationary phases chemically bonded to an inert support material. This is contrasted to classical liquid-liquid chromatography (LLC) stationary phases in which organic liquids are mechanically held to the support. Although the retention in BPC and LLC are governed primarily by partition mechanisms, many practical differences exist that permit wider application of BPC systems.

The main advantage of BPC over LLC is that BPC columns are more stable because the stationary phase is chemically bonded and not easily removed during use. This obviates the necessity of column presaturation and allows the use of solvent gradients in the mobile phase without changing the composition of the stationary phase. A wider variety of

organic stationary phases (both polar and non-polar) can be used in BPC which allows both normal and reversed phase chromatography to be carried out. Reversed phase BPC, where the mobile phase is more polar than the stationary phase, is usually achieved by bonding hydrocarbons of various chain length to the stationary phase and using this in conjunction with organically modified aqueous mobile phases. The resulting separation is usually based on the molecular weights of analyte molecules and the types of functional groups present on them which affect their relative solubilities. Alkyl-substituted BPC packings provide the broadest utility of all packings for compounds with a molecular weight less than 3000 Daltons, and should be the packing of choice for new analytes.

Properties of the bonded-phase. Bonded-phase packings are prepared almost exclusively by attaching the bonded-phase to silica-based supports through reactions of surface silanol groups (111). These reactions result in silicate esters (112), silicon-carbon bonds (113), silicon-nitrogen bonds (114) and siloxanes (111) depending on the functional groups of the bonded-phase desired. The most widely used BPC packings are based on siloxanes (Si-O-Si-R) prepared by reacting silanol groups on the support with either organochlorosilane or organoalkoxy-silane. The bonded-phase can be either monomolecular or a polymerized multilayer coating. The stoichiometry of these reactions has been extensively studied (111) and the use of monomolecular or polymeric coatings depends on the type of support particle used. Low surface area pellicular supports may require polymerization of the reactants to achieve an adequate volume of bonded phase whereas monomolecular coatings are usually adequate for porous particles.

The degree of support material coverage depends on several factors, including the type of support (pellicular or porous) and the molecular volume and chain length of the bonded-phase modifier (115). The degree of coverage can readily be estimated by treatment with trimethylchlorosilane ("capping") which should not increase the carbon content of the packing or change its chromatographic characteristics if the surface had already been completely covered.

Aside from the effect on support coverage, the chain length and structure of the bonded-alkyl group is the main factor in determining relative analyte retention. The log k' values for a given mobile phase increase linearly with hydrocarbon chain length in the absence of pore blocking effects by bulky bonded-phases (116). There also appears to be a linear relationship between log k' and the weight percent of organic coverage, independent of chain length (117). The sample loadability for reversed-phase BPC is also increased by increasing chain length although this relationship is nonlinear (116).

The exact retention mechanism for BPC packings is not definitively established although several models have been proposed. Adsorption mechanisms have been proposed in which solvent molecules compete for sites on the organic surfaces of coated particles (118). The formation of an ordered liquid phase similar to a liquid crystal has also been suggested (119). Yet another mechanism has been proposed in which the organic coating interacts with molecules in the mobile phase creating a liquid phase which is defined by the coating (120). The assumption that BPC systems are equivalent to mechanically held liquid phases seems most appropriate for predicting retention behavior and should therefore be used for practical purposes.

Separation Variables

The choice of solvent systems for BPC separations is generally similar to that for LLC. The stability of the bonded-phase is limited largely by the support material and most solvents can be used between the pH range of 2.0-8.5. Hydrocarbon BPC packings appear to be the most stable chemically although buffer and ion-pairing salts have a negative effect on their stability (121). Dissolved oxygen and strongly retained sample components also contribute to loss of column efficiency and appropriate degassing and sample clean up procedures should be used to avoid this effect.

The primary factor determining the selectivity of reversed-phase BPC separations is the polarity of the solvent used. Some commonly used solvents and their relative polarities (P') are listed in Table 1-1. Methanol, acetonitrile and tetrahydrofuran are the most commonly used solvents in that order. Varying amounts of these organic solvents are generally added to water to adjust the strength of the mobile phase. The solvent strength decreases and k' values increase as the concentration and polarity of the solvent increases in reversed-phase BPC. Significant differences can be observed in separation selectivity by the replacement of one organic solvent with another, which sometimes results in a reversal of analyte elution order (122). Methanol, for example, can be used to decrease the strength of an aqueous phase without affecting the selectivity of water since both are proton donors and acceptors. Ternary mobile phases, such as methanol/dioxane/water, have also been used to provide unique selectivity for some compounds (123).

The pH of the mobile phase can greatly change the separation selectivity for ionizable analytes. Variations of pH are generally not effective for solutes that do not ionize. The retention time for a weak uncharged acid will be relatively constant with an increase in pH until the pH approaches the pKa of the acid. The retention time then decreases and levels off when the acid has become fully ionized. This is expected since only the nonionized form should partition into the hydrophobic stationary phase significantly. In contrast, the retention time of weak uncharged bases tends to increase as pH increases. Therefore, variation of mobile phase pH can be used effectively to separate structurally similar compounds with significantly different pK values.

Temperature and flow rate are somewhat less useful in determining reversed phase chromatographic selectivity (124,125). Generally, increasing flow rate or temperature causes a linear decrease in retention for solute molecules. Temperature changes can decrease mobile phase viscosity, increase solute solubility, and alter separation selectivity to some degree although adjustment of the flow rate may be useful only for providing rapid separation. Decreasing mobile phase viscosity improves separation efficiency and BPC packings are typically stable to 80°C. BPC separations are usually carried out at room temperature with low viscosity solvents, except that operation at higher temperatures may be advantageous for relatively viscous mobile phases. Temperature effects on separation selectivity are generally useful for solutes of different functionality although these effects are not very pronounced and are unpredictable. Therefore, temperature adjustment is not usually done as an initial means of adjusting selectivity.

Achieving a bonded-phase chromatographic separation

A reversed phase BPC (bonded-hydrocarbon) column should be selected first for use in a particular separation because of its broad applicability. The C_{18} packings offer the best characteristics for applications involving compounds with high water solubility and shorter chain columns (C_1) are more appropriate for strongly retained hydrophobic molecules. Using a shorter chain hydrocarbon as the bonded-phase may also improve selectivity due to the higher mobile-phase water concentrations needed for these columns. Packings with intermediate hydrocarbon chain length (i.e., C_8) represent a good compromise and are useful for the widest variety of samples. Drug analysis usually involves compounds of high water solubility and, therefore, C_{18} columns are generally the most useful for these applications.

The first choice of mobile phase is either methanol/water or acetonitrile/water and the optimum composition is usually found empirically. A good starting point is 1:1 organic modifier/water, and concentration adjustment should be made according to the elution of sample components. A different solvent should be used primarily to alter selectivity since different concentrations can effectively adjust solvent strength.

The pH of the mobile phase is chosen either for selectivity or the suppression of ionization by protonation or deprotonation. Selectivity can be drastically affected throughout the pH range in which components are partially ionized. This partial ionization can also lead to band tailing so buffering sample components in the nonionized form is a better alternative if adequate separation can be achieved.

Gradient elution systems which normally increase the concentration of organic modifier with time are useful for samples with widely different components. Qualitative screening information can also be obtained through gradient elution.

The temperature chosen for reversed phase separations are generally more efficient between 50 and 60°C which provides about twice the number of theoretical plates than that obtained at ambient temperatures. Column stability is greater at ambient temperatures, however, and higher temperatures should be avoided when possible.

The optimum mobile phase flow rate is usually between 1-2 mL/min but depends on the type of mobile phase, the type and size of packing material, the operating temperature and the internal diameter of the column. Generally, the best flow rate is one in which the components elute quickly without losing resolution and the back pressure is maintained below 4,500 pounds per square inch.

The amount of sample injected should be less than 50 µg for pellicular BPC packings and less than 500 µg for porous BPC packings. Sample volume should not exceed one-third the retention volume of the first peak of interest although larger volumes may be tolerated for injection solutions of lower solvent strength.

Ion-Pair Liquid Chromatography

Ion-pairing has been used in liquid-liquid extraction procedures for many years, although its application to HPLC has been rather recent. The advantages of ion-pair chromatography (IPC) arise from comparison with ion-exchange methods. Ion-exchange HPLC columns are less efficient, less reproducible, less stable and available in less variety than other types of columns. The limited column variety results

in a poor potential for selectivity in ion-exchange as compared with IPC methods. These advantages have been described by Schill and coworkers (126) who have contributed greatly to the understanding and acceptance of the method.

In IPC separations, the stationary phase usually consists of the silanized silica packing normally used in reversed-phase BPC chromatography. The mobile phase consists of an aqueous buffer, organic modifier and an added counter-ion of opposite charge to the ionized sample molecule. The hydrophobic character of these ion-pairs causes the ion-pair to be more strongly retained than the free sample ion and better resolved from hydrophylic interfering substances in the sample (127).

The simplest case of IPC can be described by assuming that the sample and pairing ion are soluble only in the mobile phase, whereas the ion-pair formed is soluble only in organic stationary phase. This would result in a distribution between the phases described in equation (23):

$$RCOO^-_{aq} + IP^+_{aq} = RCOO^- IP^+_{org} \qquad (23)$$

where $RCOO^-$ represents the analyte ion and IP^+ corresponds to the pairing ion. The subscripts "aq" and "org" refer to the aqueous and organic phases, respectively. The extraction constant E can then be defined in terms of concentrations of species as follows:

$$E = \frac{[RCOO^-IP^+]_{org}}{[RCOO^-]_{aq}[IP^+]_{aq}} \qquad (24).$$

The extraction constant for a particular system depends on the temperature and composition of the mobile phase and can be related to the capacity factor k' as follows:

$$k' = \frac{V_s}{V_m} \quad E[IP^+]_{aq} \tag{25}$$

where V_s and V_m are the retention volumes of the stationary and mobile phases. In the absence of other effects, the retention of singly charged sample compounds is proportional to the concentration of the ion-pairing agent (128). The variation of $[IP^+]$, therefore, provides an additional means of controlling apparent solvent strength and can be used for differential elution of sample components.

In addition to the ion-pair concentration parameter, separation selectivity can be changed by varying the pH. Also, IP concentration gradients can be employed to further control selectivity and analysis time. Positively charged sample compounds can be similarly influenced with negatively charged pairing ions and amphiprotic molecules can be paired with either negative or positive pairing agents. This great diversity of IPC techniques combined with the separation variables already described for bonded-phase HPLC makes it possible to tailor a separation for optimum results. The application of IPC to the control of separation has been extensively described (126-133) and this introduction discusses general considerations without reference to specific applications.

Considerations Involved in Ion-Pair Separations

Some investigators have proposed that the pairing-ion is adsorbed onto the organic stationary phase causing retention of sample ions through ion exchange mechanisms (128). This suggests that the simple model proposed in equation (23) is not completely adequate to describe the processes involved, although the simple model is sufficient to

predict separation parameters for practical purposes. Adsorption of ion-pairing agents onto the stationary phase may also change the separation characteristics of bonded-phase packing materials by adding to the apparent hydrocarbon content of the stationary phase. The practical consequence of both of these phenomena is that columns must be thoroughly flushed with counter-ion containing mobile phase so that an equilibrium coating can be achieved. Very large counter-ions are more susceptible to adsorption and must, therefore, be equilibrated for a longer time.

Another problem in the use of ion-pairing agents is the need for buffer salts to control the pH of the system. This can reduce the formation of ion pairs, and consequently solute k' values, through a competing secondary equilibrium between the buffer ion and the pairing-ions (134). Those buffer ions with the same charge (cationic or anionic) as the sample ions have the largest effect. The effect also varies with the type of buffer ion. In an IPC separation of sample anions, k' values were decreased by the effect of secondary ions in the sequence $NO_3^- > Br^- > Cl^- > SO_4^{-2}$ (133). Traditional buffers such as citrate and phosphate have been used for IPC separations although they also affect k' values. The ion-pairing agent itself provides an adequate buffer in some cases.

The relative efficacy of different ion-pairing agents in increasing k' values depends primarily on two factors, the formation constant of the ion pair and the size of the counter-ion. The counter-ion functional group can have a significant effect on the ion-pair formation constant. Octane sulfate has been shown to be less efffective in ion pair formation than octane sulfonate for increasing the retention of

cations in reversed phase HPLC (135). Similar effects can be expected for different anionic pairing agents although tetraalkylammonium ions have been used almost exclusively. As expected, larger counter-ion molecules result in larger k' values in reversed phase IPC and k' values have been increased 10^5-fold when the counter-ion was varied from tetraethylammonium to tetrapentylammonium (130). The increase in log k' with total carbon number is linear although structural changes in the counter-ion such as those for surface acting pairing-ions will cause deviations from linearity. This is due partially to the concomitant ion-exchange and stationary phase altering mechanisms mentioned previously. The ion-pair formation constant may also be affected by the structure of the pairing-ion.

Solvent strength or polarity factor (P') can be adjusted in IPC by the use of organic modifiers (i.e., methanol and acetonitrile) in the mobile phase although solvent polarity does not follow the normal sequence illustrated in Table 1-1. This is because solvent strength in

Table 1-1. Solvent strength in reversed-phase BPC.

Solvent	P'
Water	10.2
Dimethyl sulfoxide	7.2
Ethylene glycol	6.9
Acetonitrile	5.8
Methanol	5.1
Acetone	5.1
Dioxane	4.8
Ethanol	4.3
Tetrahydrofuran	4.0
1-Propanol	3.9

IPC is also a function of the solvent's ability to stabilize ion pairs whereas solvent strength in BPC is mainly due to the solubility of polar nonionic solute molecules. The solvent strength in IPC should, therefore, be a function of both solvent polarity (P) and solvent dielectric strength (ε) and can be predicted by the solvent function ($P + 0.25\ \varepsilon$).

Capacity factors are greatly affected in IPC by changes in pH and are usually accompanied by large changes in separation selectivity. Maximum k' values in reversed phase IPC are observed at pH values where sample compounds are completely ionized. However, organic acids that are retained equally for example at pH 3 may be readily separated at pH 4 due to differences in their pKa values. Thus, pH variation offers a powerful tool for changing separation selectivity in IPC although this method is not appropriate for simple solvent strength adjustment. Capacity factor versus pH curves in reversed phase IPC will show the typical sigmoid relationship if the unionized sample ions are not retained by the stationary phase but the curves become more complex if this is not the case (133).

Temperature and flow rate variations follow the same general trends discussed for reversed phase BPC. The effect of temperature, however, is somewhat more important since mobile phases used in IPC are more viscous and changes in selectivity appear to be more pronounced (133). Therefore, temperature can be an important variable for optimizing separation selectivity in some IPC applications.

Aside from the special considerations discussed here, the design of an IPC separation should proceed in the same manner as that for BPC. Special precautions must sometimes be taken to prevent band tailing due

to the dissociation of ion pairs in the organic phase. This can be overcome by either increasing the counter-ion concentration or choosing another IPC system. An added benefit of IPC is that UV-absorbing counter-ions can be used to increase detectability of some compounds by allowing sample bands to leave the column as detectable ion-pairs. This may obviate the need for derivatization and both the picrate (136) and 2-naphthylsulfonate (137) ions have been used for this purpose.

A brief description of the basic analytical and clinical aspects involved in the following research has been presented here. It should be apparent from this introduction that RTP analysis of urinary PABA from patients receiving bentiromide may provide more analytically selective test. Limits of detection for PABA by RTP have been observed in the picomolar range (138) and RTP has been used to quantify PABA in vitamin tablets (139). The method is potentially adaptable to analysis of biological fluids and should prove to be more convenient than existing methods.

The potential also exists to differentiate physiological non-specificity observed in the bentiromide test by establishment of PABA metabolite concentration patterns. If either hippurate synthesis or acetylating capacity is deficient in liver patients, false positive tests due to impaired liver function may be detected.

These are goals of the following research and attempts will also be made to incorporate PABA metabolite differentiation into the RTP analytical format. A study involving the structural dependence of RTP signals from the various PABA metabolites will also be undertaken.

CHAPTER 2
EXPERIMENTAL

Apparatus

Room temperature phosphorescence measurements were made with a modified spectrophotofluorimeter (Aminco Bowman model SPF100) equipped with a rotating can phosphoroscope (140), high voltage ratio photometer (American Instrument Co., Silver Springs, MD) and a Hamamatsu IP21 photomultiplier tube (Whatman, MA). A laboratory constructed multiple solid sample bar described by Ward et al. (141) was used with a modified sample compartment lid, and a 150 W xenon arc lamp provided excitation energy. An X-Y recorder (Allen Datagraph, Inc., model 715, Salem, NH) was used to record RTP spectra, and a strip chart recorder (Model SRG, Sargent-Welch Scientific Co., Skokie, IL) was used to record decay histograms. Heavy atom and analyte solutions for RTP were spotted by use of an SMI adjustable volume (1-5 µL) micropetter (Emeryville, CA), and a dry heating block (Lab-line Instruments Inc., Melrose Park, IL) was used for alkaline hydrolysis and enzymatic incubation. Acid hydrolysis was carried out in a boiling water bath heated by a Corning model PC351 hot plate stirrer (Corning Glass Works, Corning, NY) laboratory warmer.

The pH measurements were made with a Markson pH meter (Markson Science Inc., Del Mar, CA) and ultraviolet spectra of analyte compounds were obtained using a Beckman model 25 spectrophotometer (Beckman Instruments, Irvine, CA). Visible spectra and analytical absorbance

measurements for colorimetric analysis were also made on the Beckman spectrophotometer.

A Waters Model M-6000 A liquid chromatograph (Milford, MA) equipped with a Rheodyne 7105 loop injector and 254 nm fixed wavelength detector was used for chromatographic analysis. Chromatography was performed on a Waters μ Bondapak (30x0.4 cm, 10 μm) column and an Omniscribe model 3532 strip chart recorder (Houston Instruments, Austin, TX) was used. The column temperature was controlled with an Altech water jacket (Deerfield, IL) connected to a Temptrol 153 (Precision Scientific, Chicago, IL) circulating water bath.

Materials

"Nanopure" de-ionized water (Barnstead system of Sybron Co., Boston, MA) and absolute ethanol (U.S. Industrial Chemical Co., New York, NY) were used for RTP analyte dilution. Para-aminobenzoic acid, p-aminohippuric acid (PAH) and p-acetamidobenzoic acid (PAABA) were all purchased from Sigma Chemical Co. (St. Louis, MO). Para-acetamidohippuric acid (PAHA) was synthesized as described in the following section. Diethylaminocellulose (DE-81) anion-exchange filter paper (Whatman Chemical Separation Inc., Clifton, NJ) and S&S 903 (Scheilcher and Schuell Inc., Keene, NH) were used as RTP solid-support materials. The S&S 903 filter paper was treated with diethylene triamine pentaacetic acid (DTPA) obtained from Sigma Chemical Co. as previously described (142). Silver nitrate (Mallinckrodt Chemical Works, St. Louis, MO), potassium iodide (Fisher) and thallium (I) nitrate (PCR Inc., Gainesville, FL) were used as heavy atom perturbers. Anhydrous monobasic potassium phosphate (Mallinckrodt Chemical Works, St. Louis, MO), sodium hydroxide, phosphoric acid and sulfuric acid (all obtained

from Fisher Scientific Co., Fair Lawn, NJ) were also used in RTP analysis.

Analytical reagent grade ammonium sulfamate (Eastman Chemical Co., Rochester, NY), N-(1-napthyl)ethylenediamine dihydrochloride (Eastman), concentrated hydrochloric acid (Fisher) and sodium nitrite (J.T. Baker Chemical Co., Phillipsburg, NJ) were used as reagents in colorimetric analysis.

Methanol and acetonitrile were used as organic modifiers in chromatographic analysis and were HPLC grade from Fisher Scientific Co. (Fair Lawn, NJ). The water used for HPLC mobile phases was distilled and deionized by a Watts model M (Lawrence, MA) water purifier. Cationic ion-pairing agents (tetramethylammonium chloride, tetraethylammonium chloride, tetrabutylammonium chloride and hexadecyltrimethylammonium bromide) were all 95% pure from Fisher Scientific Co. (Fair Lawn, NJ) and used without further purification. The β-glucuronidase used for pretreatment of urine samples from clinical subjects was obtained from Sigma Chemical Co. (St. Louis, MO) and contained 570,000 "Fishman" units/gm of solid. A "Fishman" unit is defined as that quantity of enzyme that will liberate 1.0 μg of phenophthalein from phenolphthalein glucuronide per hour at 37°C.

Hexane sodium sulfate, 1-octanesulfonic acid, octane sodium sulfate, decane sodium sulfate, and dodecane sodium sulfate (all from Eastman Chemical Co., Rochester, NY) were used as anionic pairing agents. All other chemicals used in chromatographic analysis (monobasic potassium phosphate, phosphoric acid, sodium acetate, acetic acid, and sodium hydroxide) were analytical grade from Fisher Scientific Co.

Synthesis of p-Acetamidohippuric Acid

Para-acetamidohippuric acid was synthesized by the reaction of acetic anhydride (Fisher Scientific Co.) with p-aminohippuric acid as described by Vogel (143). The reaction is diagrammed in Figure 2-1 and proceeds by a mechanism in which the amine group on PAHA nucleophilically attacks a carbonyl carbon atom on acetic anhydride. Half of the anhydride appears in the acyl product and the other half forms a carboxylic acid which is dissociated at the pH of the reaction mixture.

The reaction was carried out by the addition of 5.0 g of PAHA to a mixture of 60 mL H_2O and 12.5 mL of concentrated hydrochloric acid. Three milliliters of acetic anhydride along with 3.85 g of sodium acetate were dissolved in 12 mL of H_2O and added to the mixture. The combination of sodium acetate and HCl provided an initial pH of 4.2 which kept PAHA primarily in its anionic form to prevent reaction of the carboxyl group with acetic anhydride. This mixture was allowed to react at 25°C while stirring until the precipitation of white crystals which corresponded to acetylated PAHA was complete (about 2-3 minutes). The crystals were filtered and washed with cold water. Purity of the product was evaluated by thin layer chromatography on silica plates with Acetone/Ethanol/water (50/38/12) as the solvent. The R_f value for the product was 0.84 whereas PAHA demonstrated an R_f value of 0.72. There were no impurities visualized in either the product or starting material. No additional peaks corresponding to unreacted PAHA or other reaction products were found by HPLC analysis which provided further evidence of product purity. Melting points obtained from the starting material and the product were 204-206 and 236-238°C, respectively, and

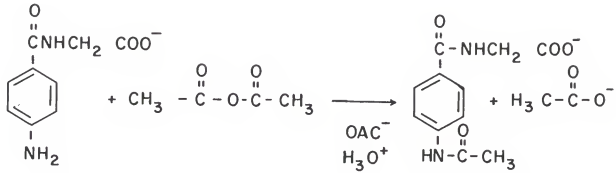


Fig. 2-1. The reaction of p-acetamidobenzoic acid with acetic anhydride to form p-acetamidohippuric acid.

the PAHA melting point was within 5° of a published result (144). Both NMR and IR spectra demonstrated peaks characteristic of the acetylated derivative (145) and were compared to spectra obtained from the starting material.

Bentiromide Administration and the Pancreatic Function Test

Subject Selection Criteria

Normal adult subjects (18 years of age or older) were determined to be in good physical health by the clinical evaluation criteria listed in Appendix II. If any of the clinical evaluation criteria indicated that disease was present, the disease must not interfere with the absorption, metabolism or excretion of PABA and be controlled by medications other than those listed in Appendix I. Normal subjects were also excluded from the study if any of the exclusion criteria listed in Appendix III were met.

Study subjects with chronic pancreatitis and exocrine pancretic insufficiency were identified by abnormal results for the secretin/cholecystokinin (CCK) stimulation test. To be considered abnormal, the maximum concentration of bicarbonate in aspirated duodenal fluid must not exceed 80 meq/L following stimulation of pancreatic secretion with 1 µg/kg of intravenous secretin and 0.02 µg/kg of intravenous cholecystokinin (146). Patients with small bowel malabsorption were diagnosed by an abnormal urinary d-xylose test and small bowel biopsy in some cases. Abnormal urinary d-xylose excretion was taken to be less than 5 g/5 hr after administration of a 25 g oral dose (147). Patients with liver disease were chosen on the basis of abnormal liver function tests and were evaluated to identify their underlying liver disorder by liver biopsy. These patients were not

evaluated by the CCK or d-xylose tests and can not necessarily be considered to be free of small bowel or pancreatic dysfunction.

Bentiromide Administration and Urine Collection

To each of 24 healthy adult volunteers, 5 patients with small bowel malabsorption, 6 patients with chronic pancreatitis, and 3 patients with exocrine pancreatic insufficiency, 500 mg of bentiromide (Adria Labs., Inc., Columbus, OH) were orally administered in 250 mL of water after an overnight fast. Just before dosing, the subjects were instructed to empty their bladders and consume 500 mL of water. An additional 500 mL of water was given and subjects remained fasting until the test was completed. Urine was collected and pooled for 6 h after drug administration, the total volume was measured and the specimen was mixed, divided into aliquots and stored at -4°C until analysis by both colorimetric and RTP methods.

An additional 5 healthy adult volunteers (4 male, 1 female) were administered bentiromide as above although separate urine samples were collected by complete bladder emptying 30 minutes, 1, 2, 3, 4, 5 and 6 hours after receiving the dose. This was done to provide PABA metabolite pharmacokinetic data. Samples were analyzed by HPLC. An extensive metabolic pharmacokinetic study was carried out on one of these subjects in which urine samples were collected at 15 minute intervals for the first 3 hours, 30 minutes intervals between 3 and 5 hours and hour intervals between 5 and 8 hours post dose.

Bentiromide was administered similarly to 10 normal volunteers, 10 patients with pancreatic insufficiency and 5 patients with various liver disorders to evaluate urinary PABA metabolite concentration patterns. In these patients, urine was collected for both a 0-3 and a 3-6 hour

period, pooled and aliquoted so that potential differences in metabolism between these populations could be more readily observed.

Colorimetric Analysis of p-Aminobenzoic Acid in Urine

Sample Preparation and Color Development

A 1.0 mL aliquot of sample urine was added to 9.0 mL of hydrochloric acid (1.3 M) in appropriately labeled 16x150 mm screw capped tubes for acid hydrolysis. The tubes were mixed and the caps were replaced loosely. All tubes were then placed in a vigorously boiling water bath and the bath was allowed to return to a vigorous boil. The screw caps were tightened and the hydrolysis was timed for 1 hour after tightening the cap. After 1 hour, the tubes were removed and allowed to cool at room temperature for about 10 minutes. The hydrolysate mixture was then diluted according to the protocol outlined in Table 2-1 so that PABA concentrations would be within the range of the standard curve. The amounts of hydrolysate and water added were chosen to maintain a total volume of 5 mL and the total dilution factor

Table 2-1. Dilution protocol for urine hydrolysate mixtures.

Urine Collection Volume mL	Hydrolysate Aliquot µL	Added Water mL	Total Dilution Factor (D)
100-200	100	4.9	500
201-500	200	4.8	250
501-1500	500	4.5	100
>1500	1000	4.0	50

(D) accounts for the 10-fold dilution involved in the hydrolysis step. Sodium nitrite (0.5 mL of a 1 g/L solution in 1.3 M HCl) was then added to each of the 5 mL hydrolysate dilutions and to 5 mL of aqueous PABA standards (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L). These solutions were mixed and allowed to stand at room temperature for exactly 4 minutes at which time 0.5 mL of ammonium sulfamate (5 g/L) was added. The tubes were mixed again and allowed to stand for 4 minutes at which time 0.5 mL of a solution containing 1 g/L of NEDA (Bratton-Marshall reagent) was added. Color development proceeded for 10 minutes after mixing the final solution and the absorbance was determined at 550 nm against a distilled water reference.

The absorbance values of standard solutions were plotted against their concentrations and the concentration of PABA in the urine hydrolysates were determined from this standard curve. The percent of the bentiromide dose recovered as PABA can be calculated for comparison to normal values by the following expression:

$$\% \text{ Recovered} = \frac{2.95\ [\text{PABA mg/L}](D)(V)}{(\text{bentiromide dose mg})} \qquad (1)$$

where D is the urine hydrolysate dilution factor and V is the total urine collection volume in mL. The constant of 2.95 is derived from the inverse of the fraction of the molecular weight of bentiromide which is contributed by PABA. This factor converts measured PABA back to an equivalent amount of bentiromide and also includes factors for conversion of μg to mg and fraction to percent.

A hydrolysis control consisting of 320 mg/L PAABA was taken through the entire analytical procedure each time assays were carried out. This

concentration of PAABA is such that 250 mg/L of PABA would be liberated if hydrolysis were complete and results were considered acceptable if PABA recovery was between 238 and 250 mg/L. This corresponds to a range of 95 to 100% recovery of the hydrolysis control which was recommended by Adria Laboratories (144).

Determination of p-Aminobenzoic Acid By Room Temperature Phosphorimetry

The RTP procedure for the analysis of PABA was developed first by the selection of the proper support material and then optimization of conditions for aqueous PABA standards. Steps were then taken to adapt the method to the analysis of urine samples from patients undergoing the bentiromide test and the final analytical procedure was evaluated with respect to various analytical variables.

Choice of Substrate

Two substrates (DE-81 and DTPA impregnated S&S 903 filter papers) were evaluated for the urinary determination of PABA by RTP. The two substrates were compared with respect to drying characteristics, pH variations, heavy atom effects and various other analytical variables.

The drying characteristics of the two substrates were compared by measuring the phosphorescence intensity of aqueous PABA solutions (25 mg/L) versus time under dry nitrogen flow. A 3 µL sample of the PABA solution was spotted onto each substrate (0.25 in. diameter discs) contained in the specially constructed sample bar. The sample bar was placed in the sample compartment purged with dry nitrogen. The strip chart recorder set on a speed of 1 in./min was started immediately after the sample bar was inserted. The relative phosphorescence intensity at 428 nm with excitation at 295 nm was recorded versus time for 50 min and

each substrate was tested in duplicate. Drying characteristics on both substrates were also studied for PABA solutions containing 50% ethanol in the same manner.

The phosphorescence intensity dependence on pH was studied for each substrate by adding small amounts of H_2SO_4 and saturated NaOH to aqueous PABA solutions (25 mg/L). The H_2SO_4 and NaOH were added by dipping a small glass rod into the acid or base and transferring the adhering acid or base solution to approximately 3 mL of the PABA solution. The pH was measured by the pH meter and more acid or base was added as needed to achieve the appropriate pH. After pH adjustment, 3 µL of the PABA solution was spotted onto the filter paper disc, samples were allowed to dry for 15 min in the nitrogen purged sample compartment and phosphorescence intensities were measured at the appropriate wavelengths. The DE-81 substrate was studied over a pH range of 1.6 to 9.6 and the DTPA impregnated S&S 903 over a range of 1.4 to 12.2. Each study was carried out twice and the results from all studies were plotted on the same graph.

The effect of I^- as a heavy atom perturber was studied for each substrate to determine relative phosphorescence enhancement from both blank and standard solutions. The blank consisted of pooled urine (pH 6.4) collected from 6 fasting volunteers under bentiromide test conditions and the same urine was spiked to yield 25 mg/L of PABA to provide a standard solution. The RTP intensity of each solution was measured 10 times on each substrate with and without addition of the heavy atom. The heavy atom was added by spotting 2 µL of 1.0 M aqueous KI onto the substrate disc prior to spotting 3 µL of the test mixture. The results were compared for significance by the students t test (149).

Randomly collected urine samples from 13 nonfasting subjects were also tested for the heavy atom effect on both substrates so that potential interferences from exogenous components in urine could be evaluated. This test was carried out similarly except that only one set of measurements was made for each sample and spiked samples were not evaluated. The results were compared for significance of the heavy atom effect on both substrates by a paired t test which is appropriate for comparison within individual samples (150).

The linearity, recovery and limit of detection were also evaluated for each substrate. The linearities of the standard curves measured from each substrate were determined by spotting 3 µL of each phosphate buffered (0.01 M, pH 6.4) standard solution, covering the concentration range of 5 to 500 mg/L. The recovery was determined by taking the ratio of the slopes of calibration curves for buffered aqueous standards, that were within the linear range, to the corresponding slope in pooled blank urine at the same concentrations. The limits of detection were determined for each substrate with and without the addition of heavy atom to buffered aqueous standards. These were calculated by determining the noise level and using the appropriate calibration curve slope to calculate the concentration that would result from three times this signal.

Analysis of p-Aminobenzoic Acid in Urine

Urine samples with collection volumes of less than 500 mL were diluted with an equal volume of distilled water prior to analysis so that the concentrations were within the range of the standard curve. A 0.5 mL aliquot of urine or 2-fold dilution thereof from patients undergoing the bentiromide test was added along with aqueous standards

(400, 300, 200, 100, and 50 mg/L) of PABA to 0.5 mL of 1 M NaOH in 10x150 mm screw-capped tubes, calibrated to 5 mL. The cap was replaced loosely and all tubes were mixed and placed in the dry heating block (which was pre-equilibrated to 120°C) for 1 h. The tubes were removed from the block and allowed to cool at room temperature for 2-3 minutes. Four milliliters of approximately 1.0 M H_2SO_4 containing 1.0 M KH_2PO_4 was then added to each tube and the tubes were mixed. This mixture is a combination reagent for neutralization and buffering and must be titrated beforehand with the 8 M NaOH solution. The H_2SO_4 concentration must be adjusted according to the titration to attain a pH of 6.4 upon addition to the sample mixture containing 0.5 mL of 8 M NaOH and is done to provide an appropriate pH for RTP analysis. After addition of the neutralization-buffer reagent, the volume is adjusted to 5 mL with distilled water by diluting to the calibration mark on the tube. This allows for evaporation that may have occurred during the incubation step. After volume adjustment and mixing, 3 µL of this mixture is spotted onto a DE-81 filter paper disc which has been mounted on the sample bar and pretreated with 2 µL of 1.0 M KI. The sample bar is then placed in the phosphorimeter sample compartment and allowed to dry under dry nitrogen flow for 15 min. The relative phosphorescence intensity was measured from each position on the sample bar with the excitation and emission monochromaters set at 295 and 428 nm, respectively. The PABA concentration in urine samples was evaluated by comparison to a best fit standard curve derived from the phosphorescence intensities and concentrations of the standards. The percent bentiromide recovered can be calculated by the same method used for the

colorimetric analysis and the dilution factor (D) is either 1 or 2 depending on the collection volume.

Ion-Pair High Performance Liquid Chromatography of Bentiromide Metabolites

Urine samples from patients undergoing the bentiromide test were centrifuged and the supernatant (100 µL) was mixed with 400 µL of a solution containing 0.66 mg/L glucuronidase (156 "Fishman" units/mL) and 0.01 M KH_2PO_4 (pH 6.8). This mixture was incubated at 37°C in a dry heating block for 30 min. Following incubation, 500 µL of methanol was added to terminate the enzymatic reaction. These mixtures and untreated urine from timed specimens involved in pharmacokinetic studies were diluted with deionized water according to the volume voided. The dilution of pooled samples (0-6 hr collection period) that were treated with glucuronidase and samples from individual timed specimens that were untreated had to be carried out differently because of the concentrations involved. The corresponding dilution schemes are shown in Tables 2-2 and 2.3. The diluted urine samples were injected without further purification into the liquid chromatograph under the chromatographic conditions and instrumental settings outlined in Table 2-4. Aqueous standards containing PABA (0.13-1.0 mg/L), PAHA (0.18-1.42 mg/L), PAABA (1.63-13.04 mg/L) and PAAHA (3.23-25.86 mg/L) were injected so that no more than three sample injections were carried out before one of the standards was injected. The peak heights corresponding to each analyte were measured and the concentrations in samples were obtained by comparison to a best fit curve constructed from the peak heights and concentrations of the standards. Sample concentrations were then corrected for dilution and volume of urine voided as shown in the

Table 2-2. Dilution protocol for HPLC analysis of timed urine samples.

Urine Collection Volume mL	Added Urine µL	Added Water mL	Dilution Factor
<50	100	9.9	100
50-100	100	4.9	50
101-150	100	1.9	20
>150	100	0.9	10

Table 2-3. Dilution protocol for HPLC analysis of pooled urine samples following treatment with glucuronidase.

Urine Collection Volume mL	Added Treatment Mixture µL	Added Water mL	Dilution Factor
<100	1000	9.0	100
100-200	1000	4.0	50
201-500	1000	1.0	20
>500	1000	0.0	10

Table 2-4. Chromatographic conditions.

Parameters	Conditions
Column	μBondapak C_{18}
Mobile Phase	0.1 M TBA - methanol (90:10)
Wavelength	254 nm
Flow Rate	1.4 mL/min
Temperature	40°C
Chromatography time	18 min
a.u.f.s.	0.01
sample volume	20 μL

following:

$$\text{Amount excreted (mg)} = 1000\ (\text{concentration, } \mu g/mL)(D)(V) \qquad (27)$$

where D is the dilution factor and V the collection volume in mL. The final chromatographic conditions were achieved by investigating the effects of mobile phase pH, the types and concentration of organic modifiers , buffer concentration, types and concentrations of ion-pairing agents, mobile phase flow rate and column temperature on the resolution of PABA and its metabolites. The influence of pH on analyte retention was studied with a mobile phase containing 10% methanol and 0.01 M KH_2PO_4. The dependence of retention on pH was also studied separately in a mobile phase containing 0.01 M TBA along with the other components. The pH of the aqueous component of the mobile phase was adjusted by addition of concentrated phosphoric acid or saturated sodium

hydroxide through the range of 2.6-6.0. The individual retention times (t_R) were measured relative to the solvent front (t_0) and the capacity factors (k') were calculated at each pH and for each analyte by the following expression:

$$k' = \frac{t_R - t_0}{t_0} \qquad (28).$$

The resolution (R_s) between each analyte at a pH selected for optimum separation was also calculated:

$$R_s = \frac{(t_{R1} - t_{R2})}{1/2\ (t_{W1} - t_{W2})} \qquad (29).$$

The quantities t_{R1} and t_{R2} refer to the retention times of the two peaks in question and t_{W1} and t_{W2} refer to their corrsponding peak widths which were measured at the baseline.

The relative influence on analyte retention for methanol and acetonitrile as mobile phase organic modifiers was studied by addition of various amounts of methanol (20, 15, 12.5, 10, 8.7, 7.5 and 5 mL) and acetonitrile (15, 12.5, 10, 8.5, 7.5 and 5 mL) to a 100 mL volumetric flask and diluting to volume with 0.01 M KH_2PO_4 adjusted to pH 4.0. The influence of methanol only (20, 15 and 10 mL added to the mobile phase) was also studied in the presence of 0.01 M TBA by addition of 10 mL of 0.1 M TBA to the flask prior to dilution. The pH of the aqueous phase for this study was adjusted to 6.0. Chromatography of the compounds of interest was carried out by injection of a standard solution for each of these mobile phases and the separation parameters k' and R_s were calculated.

The effect of buffer concentration on analyte retention in an ion-pair method was studied by adding various concentrations (0.1, 0.05, 0.01, 0.005, 0.003 and 0.001 M) of KH_2PO_4 (pH 6.0) to volume in a 100 mL volumetric flask containing 10 mL of methanol and 10 mL of 0.1 M TBA. The chromatography of relevant analytes and calculation of k' values was carried out as before.

A variety of both anionic and cationic ion-pairing agents were studied for retention behavior in mobile phases containing 10% methanol. These experiments were carried out by the addition of 10 mL of a 0.1 M solution of each ion-pairing agent to a 100 mL flask containing 10 mL of methanol and diluting to volume with 0.01 M KH_2PO_4. The pH of the KH_2PO_4 buffer was adjusted to 6.0 for cationic pairing-ions and to 2.5 for the anionic ion-pairing agents. The various agents used are listed in the materials section. Relative retention was evaluated by calculation of the k' parameter for analytes chromatographed in each mobile phase.

Pairing-ion concentration effects on analyte retention were studied for TBA by adding 1, 2.5, 5, 7.5 and 10 mL of 0.1 M TBA to a 100 mL flask containing 10 mL of methanol and diluting to volume with 0.01 M KH_2PO_4 (pH 6.0). Chromatography of the analytes was carried out for each dilution of TBA and separation parameters were calculated.

The column temperature and mobile phase flow rate were varied in an effort to minimize analysis time. The mobile phase for these studies consisted of 90/10 methanol-water with 0.01 M TBA. The column temperature was varied by adjustment of the circulating water bath thermostat to 29, 40, 50, 60, and 70°C and allowing 20 minutes for column equilibration. A standard solution of analytes was injected

after equilibration and retention parameters were calculated. The flow rate of the mobile phase was adjusted to 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mL/min with the column temperatures set at 40°C and standards were injected after a 15 minute equilibration. The separation parameter k' and R_s were again calculated following recovery of analyte peaks from the chromatograph.

Analysis of Bentiromide Metabolites by Room Temperature Phosphorimetry

Separate aliquots of urine samples from clinical subjects were hydrolyzed by both acid and base hydrolysis. The base hydrolysis was carried out by the same procedure as that used for the analysis of PABA in urine. Acid hydrolysis was carried out in screw capped tubes calibrated to 2 mL by adding 0.1 mL of the urine aliquot along with aqueous standard solutions containing 400, 300, 200, 100 and 50 mg/L of PABA to 0.9 mL of 1.3 M HCl. The caps were replaced and all tubes were placed in a boiling water bath for 15 min. The tubes were removed from the water bath and allowed to cool at room temperature for 2-3 minutes. After cooling, 0.65 mL of approximately 2 M NaOH (titrated to yield a pH of 6.4 upon addition to the mixture) containing 1.0 M KH_2PO_4 was added to each tube and the tubes were mixed. The volume of each tube was adjusted to 2 mL, mixed by vortex, and let stand at room temperature until phosphorescence was measured.

The phosphorescence at room temperature was measured from both acid and base hydrolysis mixtures in the same manner as for PABA analysis except that the substrate was not treated with iodide. The concentration of liberated PABA was determined by comparison to the appropriate best fit standard curve. The amounts of PAABA and PAABA +

PAAHA excreted were evaluated as the amounts of PABA liberated from the acid and base hydrolysis procedures, respectively, after correction for dilution and urine collection volume.

CHAPTER 3
RESULTS

Development and Optimization of the Room Temperature Phosphorimetric Method

The initial phase of this project involved evaluation of two paper substrates and optimization of conditions for room temperature phosphorimetric analysis of p-aminobenzoic acid. At various points in this study a particular condition was determined to be optimal and other possibilities were eliminated from further study. After the analysis conditions were established, a hydrolysis procedure was implemented to convert PABA metabolites back to the parent compound and the method was evaluated with clinical samples. The Bratton-Marshall colorimetric method was also evaluated and compared to the RTP procedure.

The second phase of the study consisted of the development of an ion-pair high performance liquid chromatography method for PABA and its metabolites and their quantification in various populations. Again, analytical conditions and clinical results that were determined to be unsuitable were eliminated from further study. Appropriate urine collection intervals and metabolite concentration patterns were established during this study so that false positive bentiromide test results due to liver dysfunction could be detected. Finally, an RTP method for the analysis of the metabolites PAABA and PAAHA was developed for the detection of false positives due to liver dysfunction based on liquid chromatography results.

The various analytical factors involved and the rationale for selecting particular analytical conditions will be briefly discussed in this section. A more detailed discussion of the underlying concepts involved in attaining these results will be discussed in Chapter 4.

Comparison of Two Substrates

Relatively high phosphorescence intensities of PABA have been observed from the paper substrates DE-81 (151) and DTPA treated S&S 903 (142). Therefore, these supports were chosen as potential substrates for the present analysis. Room temperature phosphorescence spectra of PABA on both substrates demonstrated excitation and emission maxima at 295 and 432 nm, respectively, and the monochromators were set at these wavelengths for analytical determinations.

Drying characteristics

The drying histogram for both substrates spotted from buffered aqueous PABA solutions is presented in Figure 3-1. The histogram consisted of 3 regions, the region corresponding to the time required for the RTP signal to reach a maximum (rise time), the plateau region in which the signal remained constant, and the decay region where the phosphorescence decreased in zero order fashion. The rise time measured from the S&S 903 substrate (13.0 min) was slightly shorter that from the DE-81 substrate (14.4 min). The plateau times for DE-81 and S&S 903 were 9.4 and 17.0 min, respectively, and the decay rate was 1.8 RPI/min for DE-81 and 1.6 RPI/min for S&S 903. The addition of ethanol to the sample solution (Figure 3-2) resulted in a slight decrease in rise time (14.4 to 14.0 for DE-81 and 13.0 to 10.6 for S&S 903) and an increase in plateau time (9.4 to 13.4 for DE-81 and 17.0-18.0 for S&S 903) for both

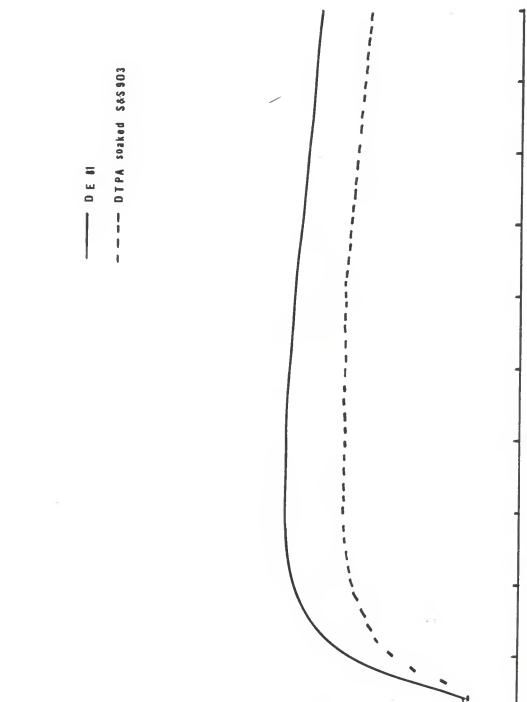


Fig. 3-1. Drying histogram for the room temperature phosphorescence of p-aminobenzoic acid adsorbed on two paper substrates.

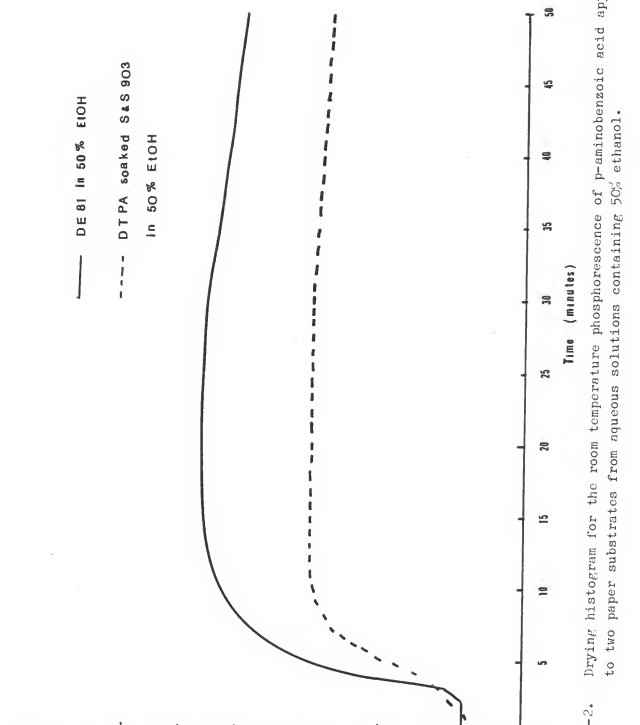


Fig. 3-2. Drying histogram for the room temperature phosphorescence of p-aminobenzoic acid applied to two paper substrates from aqueous solutions containing 50% ethanol.

substrates. The decay rate, however, was increased to 2.5 RPI/min for DE-81 and decreased to 1.3 RPI/min for S&S 903.

The rise and plateau times are important criteria since they represent the waiting time before measurements can be made and the time allowed to complete the measurements. The S&S 903 substrate is superior in this regard since short rise times and long plateau times are desirable. Although ethanol improved drying characteristics somewhat, a dilution of urine samples was required, resulting in an additional procedural step and higher detection limits for the sample. It was decided, therefore, to use aqueous sample solutions and a drying time of 15 min was chosen for further study of both substrates.

pH dependence

Figure 3-3 shows that the RTP signal of PABA from DTPA impregnated S&S 903 falls sharply below pH 3.3 and above pH 6.6. The DE-81 substrate, however, demonstrated a much wider range of relatively stable RTP intensities between pH 2.3 and 11.7. The pH of urine can vary between 4.0 and 9.0 (152) so it is important to have stable RTP intensities over this range if urine is to be analyzed without treatment. This may be necessary for metabolite analysis because of the concentrations present, and the DE-81 substrate is superior in this regard. Solutions of PABA were buffered at pH 6.4 for further study based on these results to match the pH of the pooled urine used in some of these studies.

Phosphorescence intensity and the effect of heavy atom

Iodide has been shown to be optimal for enhancement of PABA phosphorescence intensities (151) and was therefore chosen for this comparative evaluation. The RTP intensities of pooled blank urine (pH

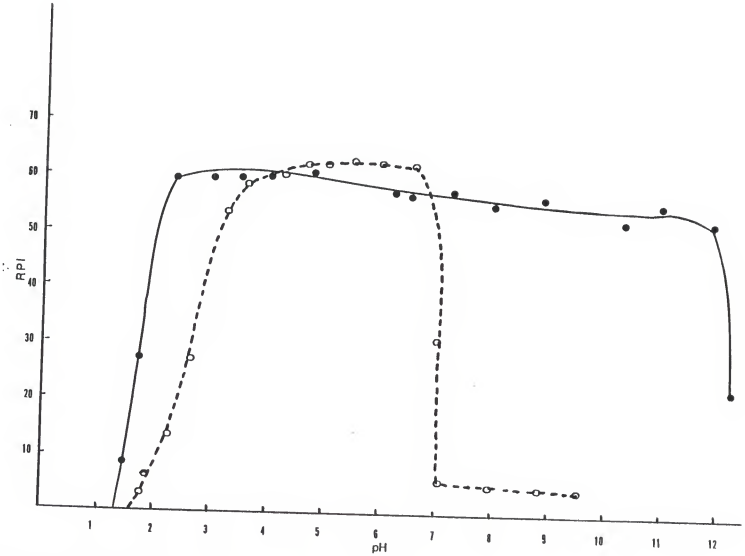


Fig. 3-3. Dependence of the room temperature phosphorescence intensity of p-aminobenzoic acid on pH for two paper substrates (DE-81 = solid line; DTPA impregnated S&S 903 = dashed line).

6.4) from fasting subjects and the same urine containing 25 mg/L PABA were compared for both substrates with and without the addition of heavy atom. The results of this study are presented in Table 3-1. The DTPA treated S&S 903 substrate demonstrated significantly greater RTP signals than DE-81 in both blank ($P<0.0015$) and spiked samples ($P<0.0025$) without the addition of heavy atom. The DE-81 substrate, however, showed a significantly greater heavy atom effect in both blank ($P<0.0025$) and spiked urines ($P<0.0025$). With the increased intensity of RTP provided by the heavy atom, DE-81 proved to be the substrate of choice in terms of potential detection limits. The higher background signal observed for the DE-81 substrate is of little consequence since these signals are only 0.8% of the analyte signal.

Table 3-1. Comparison of phosphorescence intensities on DE-81 and DTPA impregnated S&S 903 substrates with and without heavy atom.*

		Blank Urine				Urine with 25 mg/L PABA			
		No I^-		1.0 M I^-		No I^-		1.0 M I^-	
Substrate	n	Mean	S.D. RPI	Mean	S.D. RPI	Mean	S.D. RPI	Mean	S.D. RPI
DE-81	10	1.1	0.2	3.3	0.6	65.7	4.4	429.8	8.0
S&S 903 (DTPA)	10	1.3	0.4	1.7	0.4	83.5	7.7	409.3	16.0

* n = number of determinations; RPI = relative phosphorescence intensities; S.D. = standard deviation

Blank analysis of randomly collected urine samples

Potential interference from substances that may be present in urine from non-fasting subjects was evaluated and the results are shown in Table 3-2. Each sample was evaluated individually by a paired t-test which showed that RTP intensities without iodide added were significantly higher for DTPA impregnated S&S 903. The DE-81 substrate, however, demonstrated higher background when the heavy atom was added which follows the trend established for pooled urine from fasting subjects. The background in urine from non-fasting subjects was somewhat higher for both substrates with and without heavy atom which suggests that some interference from endogenous components of urine was present. The background levels with heavy atom added were still only 0.8% of the analyte signal for DTPA treated S&S 903 and 1.6% for DE-81. This indicates good selectivity of both substrates for PABA with regard to endogenous components of urine although DTPA treated S&S 903 was superior.

Table 3-2. Background analysis of randomly collected urine samples from non-fasting subjects (n = 13)

	DE-81		S&S 903 (DTPA)		Paired "t" test*
No I^-	1.3	0.3	1.5	0.3	($P<0.005$)
1.0 M I^-	7.0	4.2	3.4	1.1	($P<0.005$)

* P = probability that the difference between means occurred by chance

Linearity, recovery and detection limits

The standard curve measured from both substrates was linear over the range of 0-40 mg/L for buffered aqueous standards. The ratio of the slope of the calibration curve for PABA spotted from buffered aqueous solutions, to that spotted from urinary solution was 0.93 for DTPA impregnated S&S 903 and 0.96 for DE-81. The higher recovery from urine observed with the DE-81 substrate could be due to the wider range of pH values over which the phosphorescence signal is stable. Although both urinary and aqueous solutions were at pH 6.4, the pooled urine has a different buffer capacity than the aqueous buffer solution and the ionic character of urinary analytes may be more easily altered in the substrate environment. The observation that both substrates demonstrated less phosphorescence spotted from urinary solutions indicates there may be some competition of urinary components with PABA for either substrate binding sites or complexation with the heavy atom. The limits of detection from spotted aqueous standard solutions was 0.26 mg/L with I^- and 1.70 mg/L without I^- for the DE-81 substrate. The DTPA impregnated S&S 903 demonstrated detection limits of 0.26 mg/L and 1.30 mg/L with and without I^-, respectively.

Optimization of room temperature phosphorimetry in urine samples

The choice of the better substrate was based on the preceding studies. These studies indicated that both types of filter paper would be adequate substrates for urinary PABA analysis. The DE-81 substrate is superior in terms of pH dependence and detection limits if iodide is used. Although DE-81 shows significantly higher background in the presence of heavy atom, these levels are a small fraction of the analyte signal and would not cause interpretive interference. The DTPA

impregnated S&S 903 substrate had somewhat better drying characteristics and was superior for measurements made without iodide. An additional procedural step is required for DTPA impregnated S&S 903 since it must be prepared whereas DE-81 is commercially available. Considering all of these factors, DE-81 was chosen as the substrate for analysis of urine samples and is the only substrate used in further studies.

Although relative phosphoresence intensity from the DE-81 substrate was constant through the pH range 2.3-11.7, the optimum pH was chosen to be 6.4. This was done to approximately match the pH found in untreated urine and also approach the midpoint of the pH range in which phosphoresence intensities were relatively constant.

Having used iodide in the initial studies of heavy atom effects, various heavy atom species were investigated with the DE-81 substrate in an effort to optimize this effect. Thallium I, silver, and iodide were all compared as heavy atom species and the results are presented in Table 3-3. All relative phosphorescence intensities were normalized to the average of that found in water. The iodide perturber proved

Table 3-3. Various heavy atom effects on room temperature phosphorescence intensities of PABA from the DE-81 substrate, (n = 5).

Solvent	λ exc* (nm)	λp* (nm)	Mean RPI	SD	Normalized RPI
water	294	428	38	1.0	1.00
0.1 M KI	294	428	120	8.9	3.16
0.1 M $TlNO_3$	294	428	22	1.4	0.58
0.1 M $AgNO_3$	294	428	21	1.7	0.55

* λexc and λp = the wavelengths of maximum excitation and emission, respectively.

superior by increasing phosphorescence intensities 3.16 fold. The phosphorescence intensities in the presence of the two positively charged species (Tl^+ and Ag^+) were decreased by about 50% in each case. This is consistent with results observed for Ag^+ by Su and Winefordner (151) but inconsistent with their results for Tl^+. A possible explanation for this may be that a different counter-ion (chloride) was used for thallium in the previous study. The reduced phosphorescence intensities that were observed in this work may be due to competition of the NO_3^- anion with analyte molecules for adsorption sites on the substrate. Iodide was chosen for further study because of the higher relative intensities which provided better potential for low level detection.

The optimum hydrolysis conditions (to convert PABA metabolites back to the parent compound) were determined by comparing acidic and basic hydrolysis procedures and analysis by the HPLC procedure. The stability of PABA to hydrolytic conditions and the completeness of hydrolysis of the metabolites were used as selection criteria. Figure 3-4 and 3-5 show the time course of acid and base hydrolysis of metabolites respectively. The acid hydrolysis does not completely convert p-acetamidohippuric acid (a major PABA metabolite) to the parent compound as shown in Figure 3-5. The basic hydrolysis procedure, however, demonstrated complete hydrolysis of all metabolites to PABA which was completed in approximately 1 hour. The parent compound was stable to conditions of hydrolysis for both metabolites as indicated by a relatively constant concentration profile after the plateau region had been reached for both experiments.

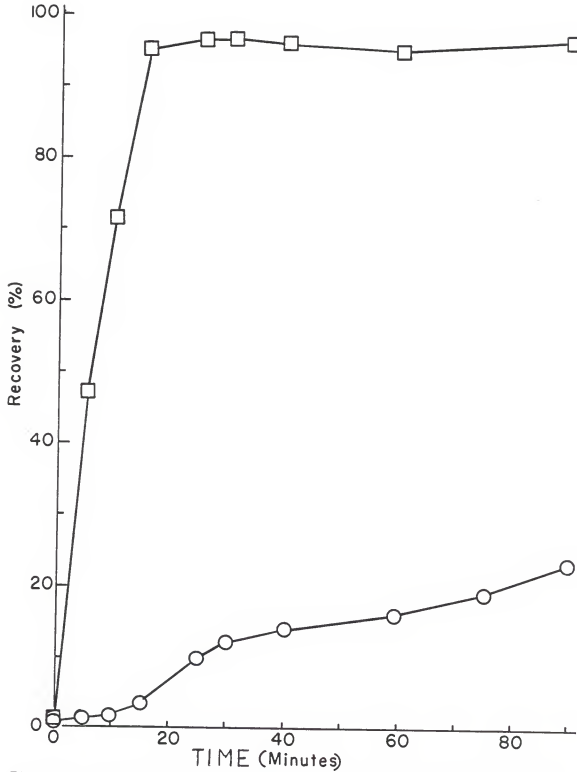


Fig. 3-4. Percent of p-aminobenzoic acid recovered versus time from p-acetamidobenzoic acid (open squares) and p-acetamidohippuric acid (open circles) under acid hydrolysis conditions.

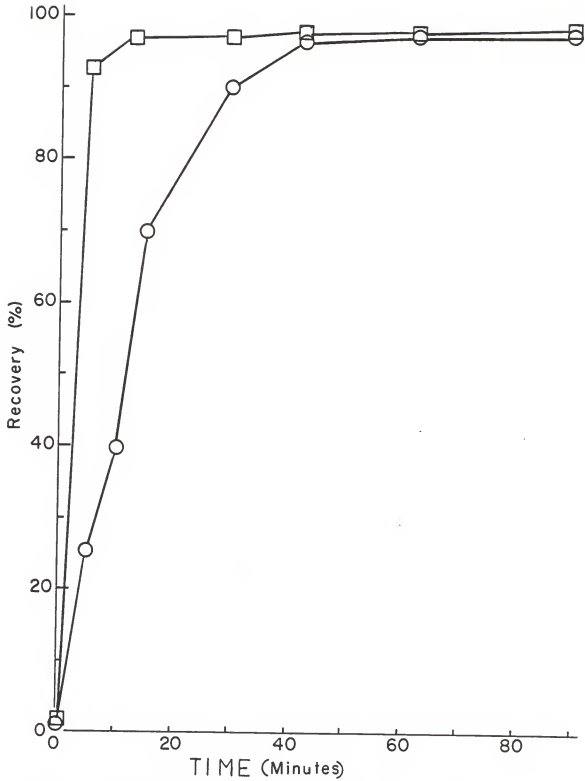


Fig. 3-5. Percent of p-aminobenzoic acid recovered versus time from p-acetamidobenzoic acid (open squares) and p-acetamidohippuric acid (open circles) under alkaline hydrolysis conditions.

At this point, it was decided to investigate why the acid hydrolysis procedure had been used successfully in colorimetric methods and experiments were carried out to explain why low recovery of PABA metabolites had not been observed previously. Para-aminohippuric acid (the primary compound to which PAAHA is converted during acid hydrolysis) was taken through the Bratton-Marshall colorimetric procedure along with an equimolar solution of PABA. The acid hydrolysis step was deleted from the procedure and the chromophores were scanned by absorbance spectroscopy (Figure 3-6). Figure 3-6 shows that PAHA apparently reacts to form a chromophore with spectroscopic properties very similar to that of the PABA-chromogen complex and although conversion to PABA was not complete, this could not be detected by the colorimetric method. The RTP method, however, is more selective for PABA and the acid hydrolysis procedure would be inadequate because of low recovery of PABA metabolites and variable PABA metabolism.

This was further investigated by recovery experiments in which PABA and equimolar solutions of PAHA, PAABA and PAAHA were taken through the

Table 3-4. Recovery of PABA and metabolites by acid hydrolysis followed by colorimetric quantification (n = 5)

	Mean concentration (mg/L)	SD	Mean percent recovered
PABA	2.48	0.03	99.2
PAHA	2.44	0.03	97.6
PAABA	2.34	0.04	93.6
PAAHA	2.29	0.02	91.7

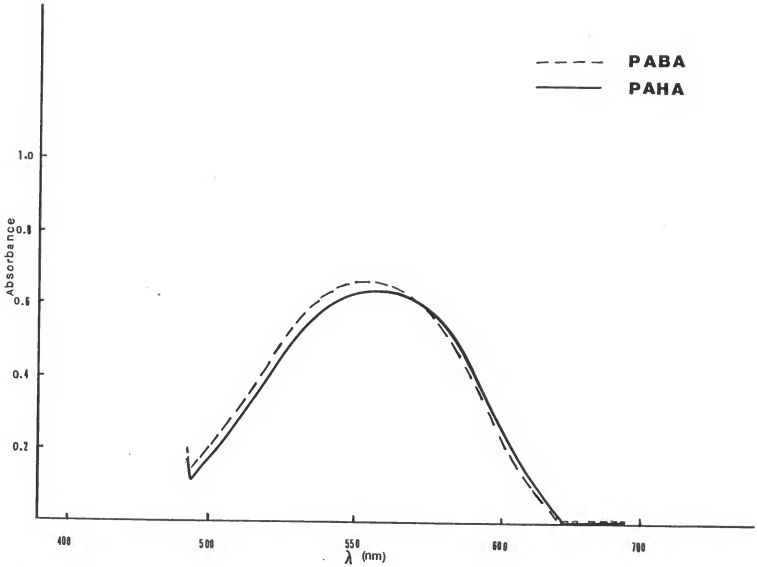


Fig. 3-6. Absorption spectrum for the Bratton-Marshall reaction productions of p-aminobenzoic acid (PABA) and p-aminohippuric acid (PAHA). Both compounds were present in the same molar concentrations (1.82×10^{-3}M) before the Bratton-Marshall procedure was carried out.

entire colorimetric procedure which included acid hydrolysis. The results of this study are shown in Table 3-4 and indicate that PAHA demonstrated the highest recovery whereas PAABA and PAAHA were successively less recovered. This is probably because of incomplete hydrolysis of the amido groups on these compounds and the fact that the amides do not react with Bratton-Marshall reagent to form a chromophore.

Clinical Evaluation of the Room Temperature Phosphorescence Method

Having developed and optimized the RTP method, studies were carried out to evaluate the method in spiked urine and in urine samples taken from subjects undergoing the bentiromide test. The method was evaluated for recovery, precision, selectivity, and various other analytical variables. The method was also compared with colorimetric analysis by correlation studies.

Analytical Recovery

Para-aminobenzoic acid (250 mg/L) and its metabolites PAHA (354 mg/L), PAABA (326 mg/L) and PAAHA (431 mg/L) were diluted in pooled blank urine, taken through the alkaline hydrolysis procedure, and evaluated by the RTP method (Table 3-5). These metabolite concentrations are such that an equivalent amount of PABA (250 mg/L) is liberated for each compound if hydrolysis is complete. Preliminary experiments demonstrated that the phosphorescence intensities produced by the metabolites studied were all less than 10% of that produced by PABA (Figure 3-7). Therefore, any appreciable signal detected from the hydrolysate solutions was due to liberated PABA and not from metabolite phosphorescence.

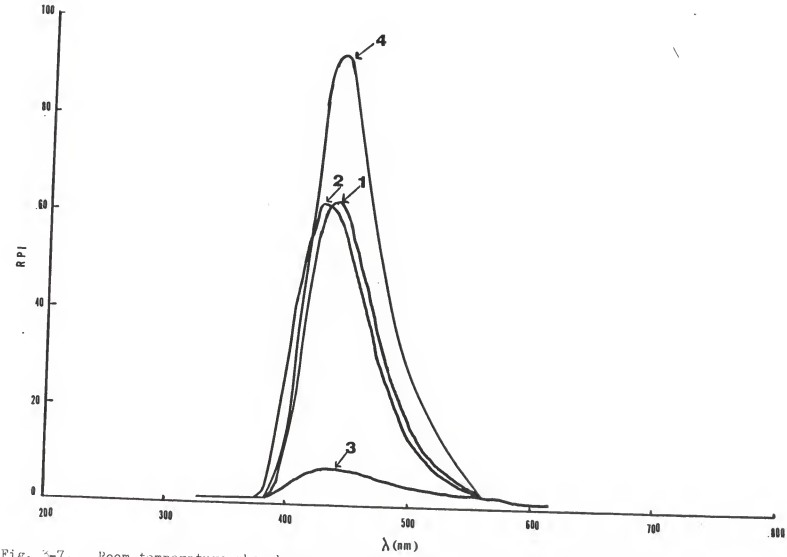


Fig. X-7. Room temperature phosphorescence emission spectra of p-aminohippuric acid (1), p-acetamidobenzoic acid (2), p-acetamidohippuric acid (3) and p-aminobenzoic acid (4) adsorbed onto the DE-81 substrate. The phosphorescence spectrum of (4) was measured at a 10-fold lower signal amplification.

Table 3-5. Analytical recovery of PABA and metabolites added to drug-free urine by room temperature phosphorescence

	n	Concentration of PABA (mg/L) Added	Mean	SD	Mean (%)	SD
PABA	10	250	253	7.1	101	2.8
PAHA	10	354	265	8.3	106	3.3
PAABA	10	326	245	4.2	98	1.7
PAAHA	10	431	244	8.9	98	3.6

Table 3-6. Precision of the room temperature phosphorescence method

Within-run

Mean (mg/L)	n	S.D.	C.V.(%)*
93.2	10	7.9	8.5
162.0	10	8.6	5.3
347.2	10	21.8	6.3

Day-to-day (mg/L)

Mean	n	S.D.	C.V.(%)
75.5	10	10.9	14.4
163.6	10	9.0	5.5
335.5	10	19.2	5.7

* C.V. = coefficient of variation.

Precision

Precision was evaluated by repeated analysis of frozen aliquots taken from patient samples on both a within-day run and day-to-day basis (Table 3-6). The three patient samples chosen provided concentrations reflecting the entire range of the assay. The coefficient of variation (CV) for all samples was less than 10% except that for day-to-day precision of the sample with the lowest concentration.

Linearity

The standard curve constructed from standards that were subjected to the alkaline hydrolysis step was linear through the range of 0 to 40 mg/L (Figure 3-8). The range of linearity is the same as that for aqueous standards that were not hydrolyzed which shows that the linear range is not affected by the concentrated electrolyte environment of hydrolysate mixtures. The linear range corresponds to original urinary PABA concentrations of 0 to 400 mg/L and covers most concentrations found in patient samples. Of the 75 patient samples analyzed, only 6 required dilution with an equal volume of distilled water prior to hydrolysis to adjust their concentrations into the range of the standard curve.

Sensitivity

The limit of detection measured with standard solutions of PABA diluted in pooled blank urine was 0.67 mg/L. This detection limit is slightly higher than that measured for iodide treated DE-81 with aqueous standards (0.26 mg/L) and is due to the higher phosphorescence intensity of the pooled urine relative to that of water. The detection limit in urine, however, is far below the level found in patient samples.

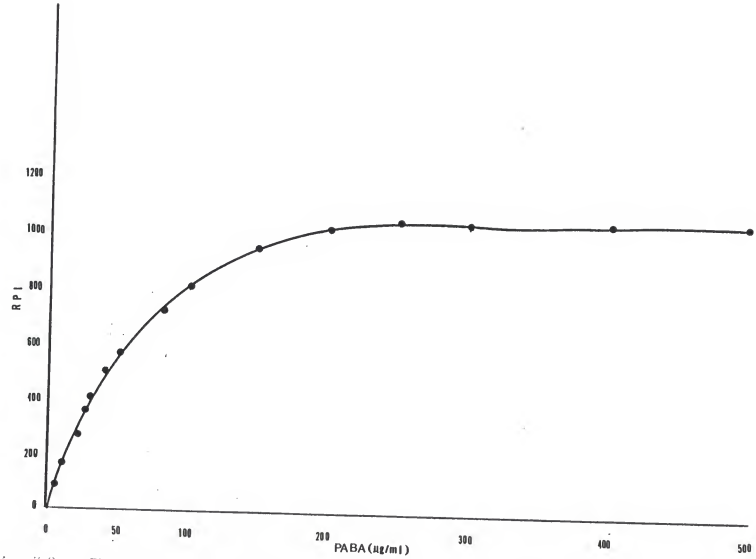


Fig. 3-8. Plot of relative phosphorescence intensity at room temperature versus p-aminobenzoic acid (PABA) concentration for the DE-81 substrate showing the linear range of the standard curve.

Selectivity

Several commonly used drugs were tested for interference with the RTP method by adding 500 mg/L of each drug to pooled blank urine and analyzing these solutions for PABA. The only drug that demonstrated an interference that would preclude clinical evaluation was procaine (Table 3-7). The consequence of the procaine interference is that patients must be screened to see if they have recently received the drug. Most of the drugs tested have been shown to interfere with colorimetric methods (see Appendix I) and this study illustrates the superiority of the RTP method in this regard. Xylose was tested because of the possibility of administering the xylose test concurrently with the bentiromide test.

Table 3-7. Drug interference study based on analysis of aqueous solutions of selected drugs (500 mg/L) by RTP.

Drug	Apparent PABA concentration (mg/L)	Percent Interference
Chlorthiazide	0	0
Sulfadiazine	0	0
Sulfamethazine	0	0
Atropine	0	0
Neomycin sulfate	0	0
Indomethacin	20	4.1
Chlorpropramide	0	0
Tolbutamide	0	0
Methochlopramide	0	0
Hydrochlorthiazide	0	0
Acetaminophen	0	0
Procaine	203	40.6
Acetyl salicylic acid	5	1.1
Lidocaine	0	0
Caffeine	0	0
Xylose	0	0
Sulfanilimide	40	8.0
Chloramphenicol	3	0.7

Patient Sample Correlation

Urinary PABA concentrations measured by the RTP method were compared with those obtained by the Bratton-Marshall colorimetric procedure. Urine samples were collected from patients undergoing the bentiromide test according to the protocol in the methods section of this work. Consequently, drug interference was not a factor. The two methods demonstrated good agreement as indicated by the correlation plot shown in Figure 3-9. Regression analysis of the Bratton-Marshall results (x) versus the RTP results (y) demonstrated a linear relationship of $y=0.997x+1.651$ for 75 clinical samples containing between 58 and 786 mg/L. The correlation coefficient (r) was 0.993 and the standard deviations of the slope and intercept were 0.002 and 0.300, respectively.

Development and Optimization of the Ion-Pair High Performance Liquid Chromatography Method

Ito and coworkers have quantified PABA by HPLC in urine samples after metabolite hydrolysis (153). The goal here, however, is to quantify individual PABA metabolites so that false positive bentiromide test results due to liver dysfunction may be detected. For this purpose, a new analytical approach which would resolve metabolites of PABA from each other and also from endogenous components of urine was necessary. After initial experiments, conducted with various organic modifiers and at different pH, it was decided to employ ion-pairing agents which would enhance the retention of the compounds of interest without affecting the endogenous components of urine. Both anionic and cationic ion pairing agents were tested since all analyte molecules are amphiprotic. The ion-pair method that was ultimately developed was then optimized and evaluated.

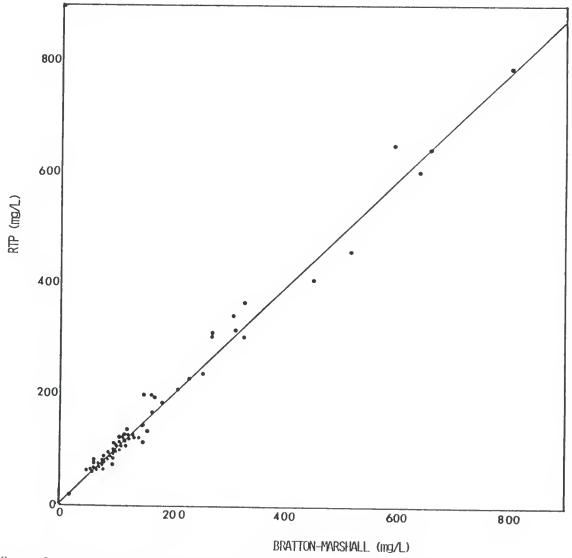


Fig. 3-9. Correlation plot of p-aminobenzoic acid concentrations in urine measured by the room temperature phosphorescence procedure (y) versus those measured by the Bratton-Marshall method (x)(y=0.997x + 1.651; n=75; r=0.993).

Organic Modifier

The influence of methanol and acetonitrile concentration on the retention of the compounds of interest was studied without the use of ion-pairing agents. The aqueous component of the mobile phase was buffered at pH 4 with a 0.01 M phosphate buffer and the column was left at ambient temperature (25° C). In all cases, decreasing the concentration of organic modifiers increased the retention of the solutes by the column (Figures 3-10 and 3-11). However, acetonitrile had a more selective effect on retention compared to methanol. For example, lowering the acetonitrile concentration from 15 to 5% increased the capacity ratios of PAABA and PAAHA from 1.0 to 2.4 and 0.4 to 1.3, respectively. The corresponding increases in the capacity factors of PABA and PAHA were from 1.0 to 1.3 and 0.4 to 0.6, respectively. Consequently, 5% acetonitrile provided satisfactory overall retention characteristics, but resolution of PABA and PAAHA was poor (Figure 3-10). In comparison, methanol demonstrated a less selective effect on the capacity factors of PAABA and PAAHA which increased from 2.6 to 4.1 and 0.9 to 2.3, respectively, when the methanol concentration was decreased from 20 to 5% (Figure 3-11). The corresponding increases in the capacity factors of PABA and PAHA were 1.0 to 1.4 for PABA and 0.4 to 0.5 for PAHA. With concentrations of acetonitrile above 5%, the solutes eluted too rapidly for analytically useful results.

The results indicate methanol is a stronger solvent than acetonitrile for PAABA and PAAHA than for their non-acetylated counterparts. A possible explanation for this is that amides (i.e., PAABA and PAAHA) are generally stronger in their hydrogen bonding ability than amines (i.e., PABA and PAHA)(154) and could be expected to

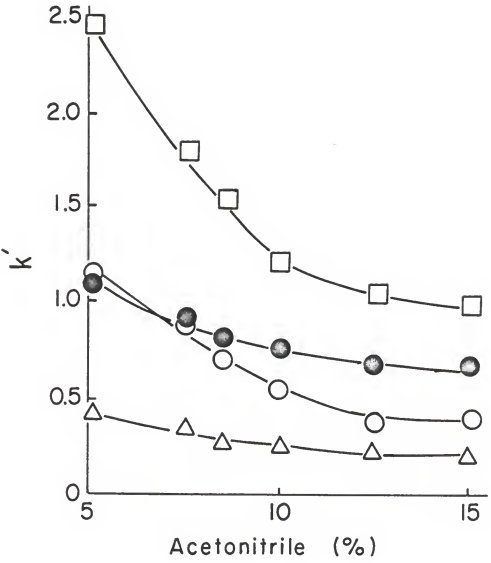


Fig. 3-10. The effect of mobile phase acetonitrile concentration on capacity factors for p-aminobenzoic acid and metabolites (p-aminobenzoic acid, closed circle; p-aminohippuric acid, open triangles; p-acetamidohippuric acid, open circles; p-acetamidobenzoic acid, open squares). The chromatographic conditions were the same as those listed in table 2-4 except the flow rate was 1.2 ml/min and the column temperature was 25°C.

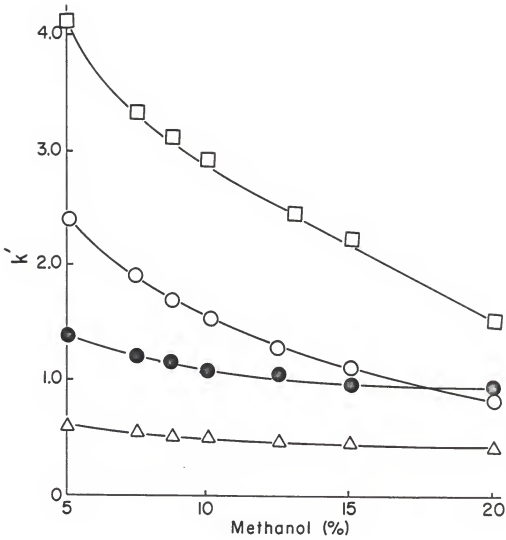


Fig. 3-11. The effect of mobile phase methanol concentration on capacity factors for p-aminobenzoic acid and metabolites. The symbols and conditions are the same as for figure 3-10.

interact more strongly with a hydrogen bonding solvent. Methanol can serve as both a proton donor and acceptor (155) making it a stronger solvent than acetonitrile for hydrogen-bonding interactions (156).

With a mobile phase containing 10% methanol the resolution of all the peaks of interest was greater than 1.00. Although a mobile phase containing 10% methanol in a phosphate buffer adequately resolved the peaks of interest, interference from endogenous components of urine was observed. Complete resolution was achieved by optimizing the effect of pH and by the addition of ion-pairing agents to the mobile phase.

pH Optimization

The dependence of retention on pH was studied with a mobile phase containing 10% methanol at ambient temperature. The pH of the aqueous component of the mobile phase was adjusted by addition of phosphoric acid or sodium hydroxide to 0.01 M KH_2PO_4. Figure 3-12 shows that the retention of all components decreased upon increasing the pH above 3.5 due to deprotonation of the carboxylic groups of the solute (157). Changes in the elution order with changing pH may be attributed to differences in the pKs of the carboxyl groups. For example, the pKa values for PABA and PAHA are 4.9 and 3.6, respectively (158). No literature values for the pKas of PAABA and PAAHA were available. These parameters were calculated from the data in Figure 3-12, however, and pKa values of 4.27±0.07 for PAAHA and 4.23±0.04 for PAABA were obtained. The pH ranges used for the pKa calculations were 3.9-4.8 for PAAHA and 3.9-4.7 for PAABA. The pKa of PAABA was also determined by an absorptiometric method as a check on the value obtained from chromatographic data and was found to be 4.18±0.05. The close

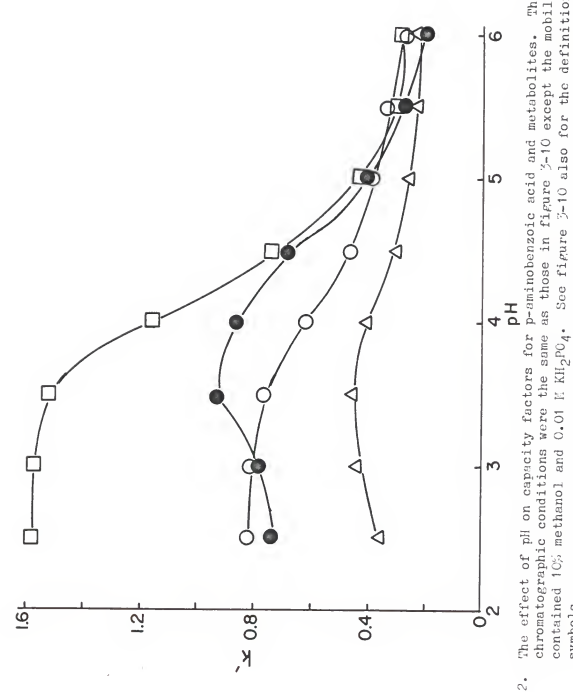


Fig. 3-12. The effect of pH on capacity factors for p-aminobenzoic acid and metabolites. The chromatographic conditions were the same as those in figure 3-10 except the mobile phase contained 10% methanol and 0.01 M KH_2PO_4. See figure 3-10 also for the definition of symbols.

comparison between the two values indicates that the pKa's measured chromatographically were reliable.

Separation was achieved at pH 4.0. Under these conditions, however, components of human urine persisted and ion-pairing agents were necessary to enhance the retention of the compounds of interest without affecting the endogenous components of urine.

Ion-pairing Agent

The increase in retention provided by ion-pair formation is dependent on the charge, hydrophobicity and size of the ion pairing agent (155). Increasing the pairing-ion alkyl chain-length for a homologous series of symmetrical counter ions should, therefore, result in an increase of log k' with increasing counter ion carbon number (160).

Various alkylammonium ion-pairing agents (0.01 M) were studied for their effect on analyte capacity factors and the results are shown in Figure 3-13. The positive deviation from linearity demonstrated for the hexadecyltrimethylammonium ion (19 carbons) is possibly due to a secondary equilibrium in which these surface acting pairing ions are adsorbed onto the surface of the stationary phase (161). This could result in alteration of the stationary phase by increasing its effective hydrophobicity and creating an apparent ion-exchange effect. Both of these factors would contribute to the observed positive deviation. Another explanation for this deviation from linearity may be that the charged head of the asymmetric hexadecyltrimethylammonium ion is more accessible for ion pairing than those of the symmetrical series represented by the other pairing ions. The homologous series of symmetrical pairing ions have the bulky alkyl groups bonded directly to

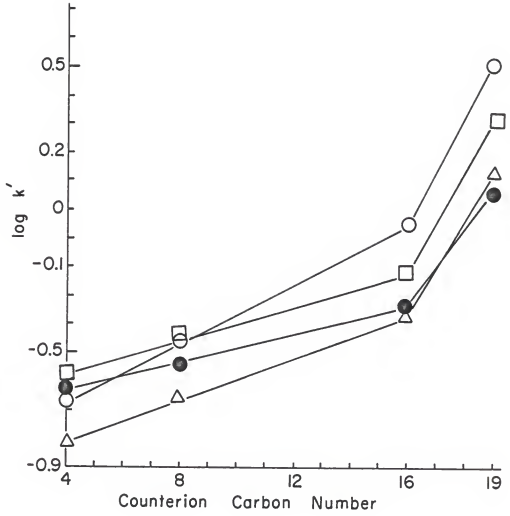


Fig. 3-13. Cationic counter-ion size effect on capacity factors for p-aminobenzoic acid and its metabolites. Tetramethylammonium chloride = 4 carbons; tetraethylammonium chloride = 8 carbons; tetrabutylammonium chloride = 16 carbons and hexadexyltrimethylammonium bromide = 19 carbons. The symbols and conditions were the same as for figure 3-10 and the pH of the mobile phase was adjusted to 6.0.

the ammonium ion which may cause steric hindrance with binding to solute ions. Increased availability of the charged head on the long chain alkylammonium ion would result in a higher ion-pair formation constant and thus a higher extraction constant (E). This would be expected to increase solute retention according to equation 25 in the introduction.

Anionic ion-pairing agents were also studied for their effect on retention of the compounds of interest with the aqueous component of the mobile phase adjusted for pH 2.5. Figure 3-14 shows a plot of analyte log k' values versus counter ion carbon number for the anionic pairing ions studied. The increase in retention for PAHA and PAAHA is approximately linear whereas log k' for PAABA and PABA show deviations from linearity above carbon number 8. The ion-pairing agents used constitute a homologous series of alkyl sodium sulfates so any deviations from linearity are probably not attributable to accessibility of the charged head on the pairing ion to a site of positive charge on the analyte. Also, these deviations occurred for both PAABA which is unionized at pH 2.5 and PABA which is partially ionized. This suggests that the deviations observed were not due to the formation of ion pairs because PAABA would not form ion pairs with the pairing agent. The most reasonable explanation is that the stationary phase was altered by absorption of pairing ions. This could result in a decrease in retention for PAABA and an increase in retention for PABA because the stationary phase would take on an anionic character which would result in stronger interactions with positively charged molecules and weaker interactions with uncharged species. This explanation is consistent with the positive deviations in retention observed for PABA and the negative deviations observed for PAABA.

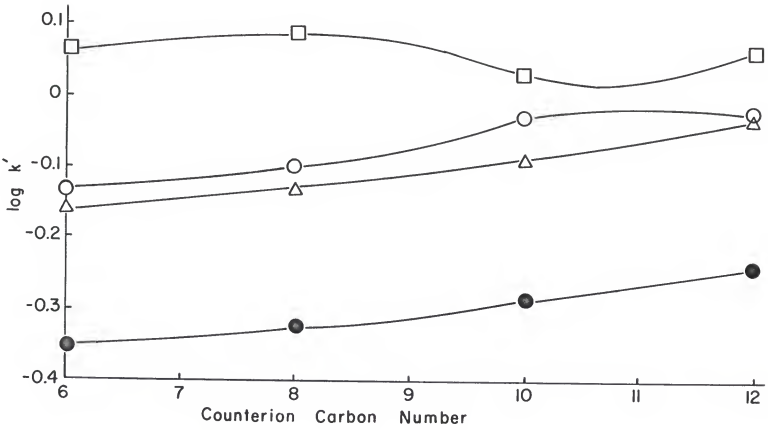


Fig. 3-14. Anionic counter-ion size effect on capacity factors for p-aminobenzoic acid and its metabolites. Hexane sodium sulfate = 6 carbons; octane sodium sulfate = 8 carbons; decane sodium sulfate = 10 carbons and dodecane sodium sulfate = 12 carbons. The symbols and conditions were the same as for figure 3-10 and the pH of the mobile phase was adjusted to 2.5.

The dependence of analyte capacity factors on pH without pairing ion (Figure 3-12) indicates that both PAABA and PAAHA are unionized at pH 2.5 whereas PABA and PAHA are at least partially ionized. A greater effect on retention should be observed for the more ionized species when the pairing ion is increased in size and the greater ion-pair effect observed in Figure 3-14 for PABA and PAHA is probably a result of this.

Octanesulfonic acid was also compared with octane sodium sulfate to determine the effect of a different charged head group on the pairing ion. The results of this study are shown in Table 3-8 for each of the analyte molecules. The retention of all solutes was increased by the substitution of the sulfonate for the sulfate suggesting a higher formation constant for the former pairing ion. The most likely reason for this observation is higher negative charge density on the sulfonate molecule which results in stronger association with the cationic pairing agent.

Table 3-8. Comparison of octane sodium sulfate with octane sulfonate for retention of PABA and its metabolites

	Octane sulfate k'	Octane sulfonate k'	Percent difference
PABA	0.79	0.83	5.1
PAHA	0.73	0.80	9.6
PAABA	1.22	1.28	4.9
PAAHA	0.47	0.60	27.7

The cationic pairing ions demonstrated greater increases in capacity factors for all analytes when compared to the anionic pairing agents. This, no doubt, can be attributed to the higher fraction of solute ions at pH 6 than at pH 2.5 (see Figure 3-12). The bonded phase column used is not stable below pH 2.5 so lower pH values could not be employed to further ionize solute molecules. Furthermore, the retention of PAHA was only slightly affected by anionic pairing and could not be resolved from fast eluting endogenous urinary components. It was decided, therefore, to use a cationic pairing agent for further study and tetrabutylammonium ion (TBA) was chosen specifically because it provided a good compromise between high capacity factors, needed for resolution from interferences, and fast analysis time.

The effect of methanol concentration on retention of the compounds of interest followed the same trend with 0.01 M TBA added (Figure 3-15) as that shown in Figure 3-11 without TBA. Again, the best separation was achieved at 10% methanol.

Pairing-ion Concentration

The effect of TBA concentration on solute retention was studied at pH 6.0 and ambient temperature in a mobile phase consisting of 10 v/v% methanol and 90% aqueous phosphate buffer. The addition of TBA (Figure 3-16) to the mobile phase resulted in a reversal of relative positions for PABA and PAHA along with PAABA relative to PAAHA, which was due to the greater ion-pair effect on the glycine conjugates. Analyte capacity factors were studied through the range of 0.001 to 0.01 M TBA and the capacity factors increased from a range of 0.11 to 0.50 at 0.001 M TBA to a range of 0.92 to 2.50 at 0.01 M TBA, without any significant change in separation selectivity. This agrees with previous studies (161,162)

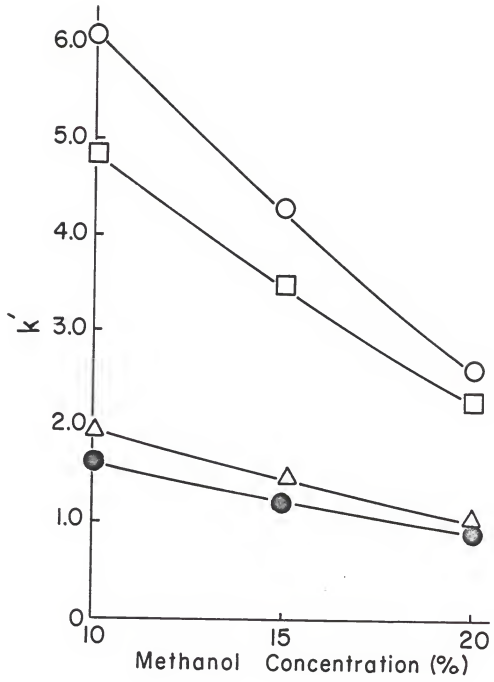


Fig. 3-15. The affect of mobile phase methanol concentration on capacity factors for p-aminobenzoic acid and its metabolites with tetrabutylammonium chloride (0.01 M) added to the mobile phase. The symbols and conditions were the same as for figure 3-10.

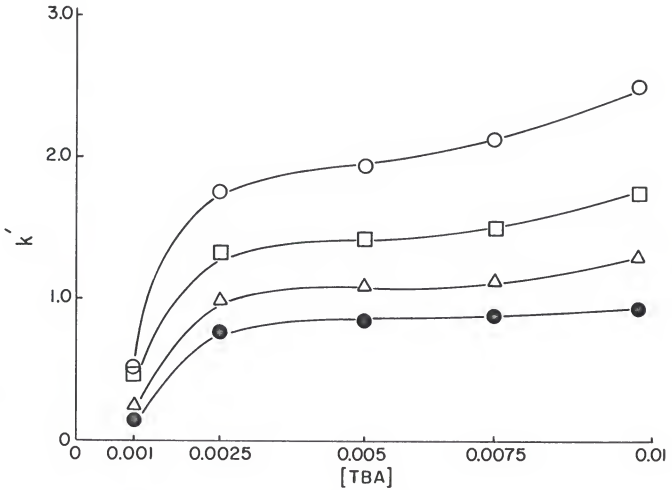


Fig. 3-16. The effect of tetrabutylammonium chloride (TBA) concentration on the capacity factors of p-aminobenzoic acid and metabolites. The symbols and conditions are the same as those for figure 3-10 and the pH of the mobile phase was adjusted to 6.0.

where it was seen that pairing-ion concentration had no effect on selectivity although solute ion retention is significantly affected. The relationships between TBA concentration and solute capacity factors are sigmoidal which has been previously observed (162). This is also consistent with low pairing-ion binding to the stationary phase since surface acting pairing-ions usually show a parabolic dependency of capacity factors on pairing-ion concentration (162). For the purpose of method development, it is advantageous to identify the maximum effect and use the pairing-ion at this concentration, which in our case was 0.01 M.

Optimization of pH With the Ion-Pairing Agent Added

The dependence of retention on pH with TBA added (Figure 3-17) was much less pronounced than that shown without TBA in Figure 3-12. The capacity factors of all compounds were increased as the pH was raised above pH 5.0 showing the increased ion-pairing effect with increasing concentrations of free carboxylate ions. Between pH 4.0 and 5.0 the relationship can best be described as a combination of ion-pairing and ion-suppression effects since the weaker acids PABA and PAABA demonstrated the greatest relative increase in capacity factors. The minimum capacity factor for all compounds occurred at about pH 5.0 suggesting a common point at which the ion-pair effect begins to dominate over the ion suppression effect. This is an interesting observation since it seems that the greater ion suppression effect of PABA and PAABA is about equally compensated by greater ion-pair effects on PAHA and PAAHA at that pH.

Separation was achieved with a resolution of 1.0 between PABA and PAHA, 1.14 between PAHA and PAABA, and 2.0 between PAABA and PAAHA at pH

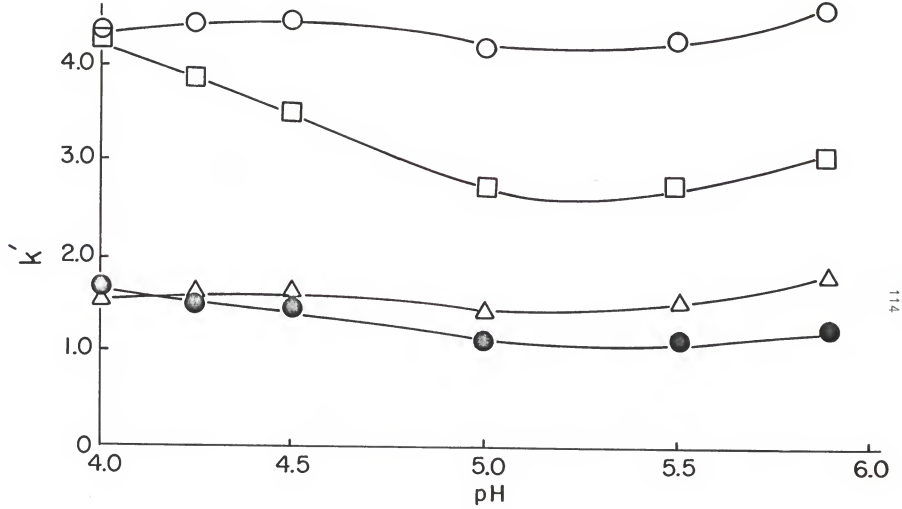


Fig. 3-17. The effect of pH on capacity factors for p-aminobenzoic acid and its metabolites with 0.01 M tetrabutylammonium chloride added to the mobile phase. See figure 3-10 for other experimental conditions and a definition of the symbols.

5.8. Addition of TBA also increased the retention of urine contaminants slightly to a capacity factor of 1.1.

Effect of Buffer Ions

Buffer ions can compete with ion-pairing agents for analyte molecules, thereby, reducing the influences of the latter (158). The effect of phosphate concentration on analyte capacity factors is shown in Figure 3-18. An average decrease in analyte capacity factors of 20% was observed between 0.0001 and 0.005 M phosphate without changing the elution order of sample components. Therefore, it was decided to eliminate the phosphate buffer at this point and use TBA alone for further study. The TBA alone provided a stable pH of 7.4 and obviated the buffer effect without affecting peak shape.

Temperature and Flow Rate

Relative separation was investigated between the temperature and flow rate ranges of 29-70° C and 1.0 to 2.0 mL/min, respectively. Plots of capacity factors versus temperature (Figure 3-19) and flow rate (Figure 3-20) show little variation because capacity factors are measured relative to the solvent front which is also affected by temperature and flow rate. Inspection of the individual chromatograms, however, allowed the optimization of variables with regard to providing fast analysis time while still maintaining separation with all resolution parameters greater than one. The optimum combination of temperature and flow rate chosen in this manner was 40° C and 1.4 mL/min. At this point, all compounds of interest had been adequately separated and resolved from endogenous components of urine. Example chromatograms from blank urine and urine spiked with the compounds of

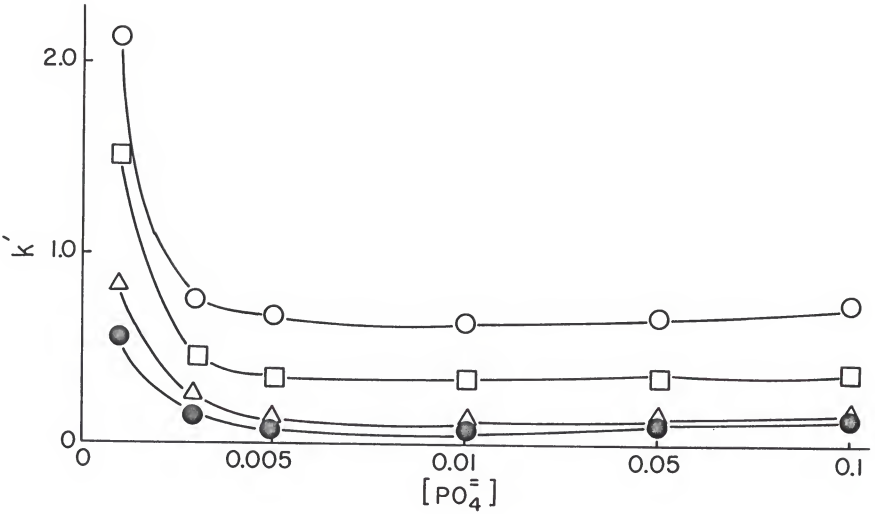


Fig. 3-18. The effect of phosphate concentration on capacity factors for p-aminobenzoic acid and its metabolites with 0.01 M tetrabutylammonium chloride added to the mobile phase. The symbols and conditions are the same as those for figure 3-10 with the pH of the mobile phase adjusted to 6.0.

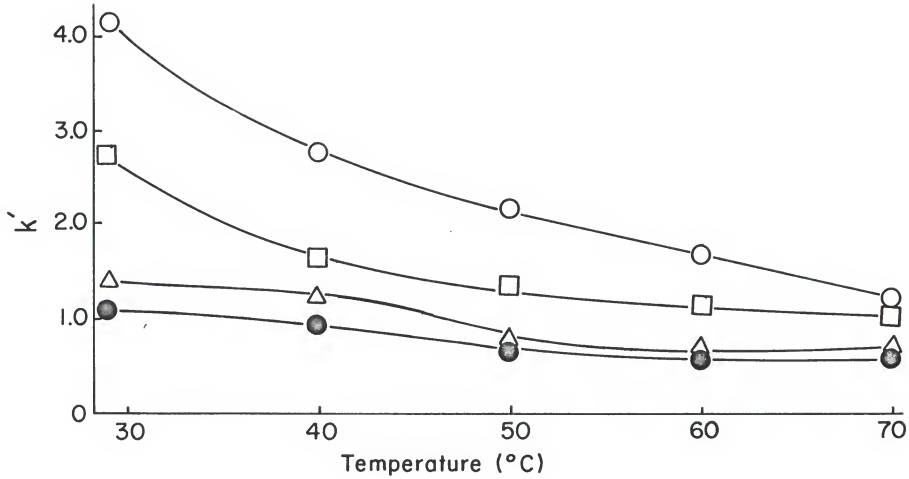
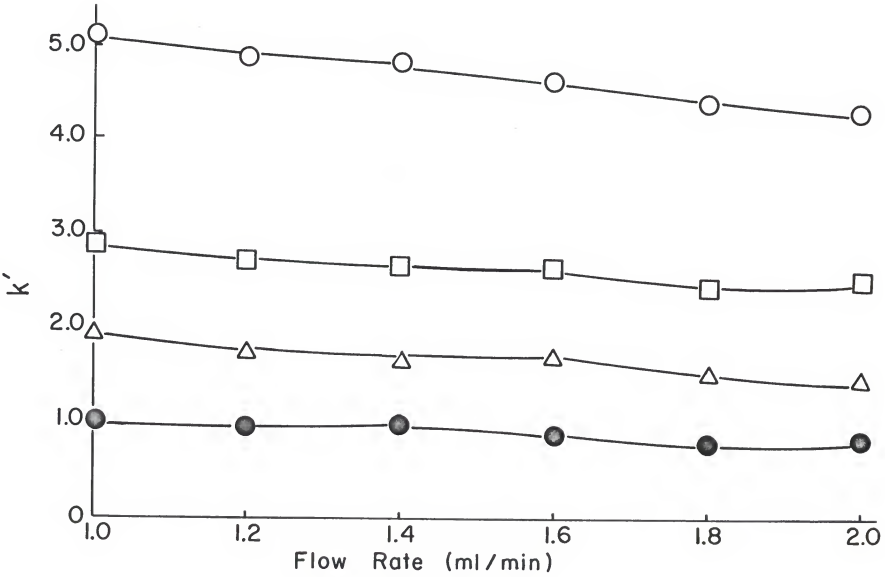


Fig. 3-19. The effect of column temperature on capacity factors for p-aminobenzoic acid and its metabolites. All other conditions and the symbols were the same as those for figure 3-10.

Fig. 3-20. The effect of mobile phase flow rate on the capacity factors for p-aminobenzoic acid and its metabolites. The symbols and conditions were the same as those for figure 3-10 except the column temperature was adjusted to 40°C.


5.0
4.0
3.0
2.0
1.0
0
k'
1.0
1.2
1.4
1.6
1.8
2.0
Flow Rate (ml/min)

interest is shown in Figure 3-21 and demonstrates the separation achieved.

Validation of the Ion-Pair Method

Linearity and detection limits. Known amounts of PABA (0.13 - 1.0 mg/L), PAHA (0.18 - 1.42 mg/L, PAABA (1.63 -1 13.04 mg/L) and PAAHA (3.23 - 25.86 mg/L) were injected in aqueous solution and subjected to the HPLC proceudre. All analytes demonstrated a linear relationship between peak height and concentration over the range of interest (Figures 3-22 and 3-23). The slope and corresponding standard error for the mean of 6 standard curves was 31.7 ± 0.44 for PABA; 17.1 ± 0.20 for PAHA; 17.7 ± 0.19 for PAABA and 8.5 ± 0.06 for PAAHA. The consistently low standard errors were a result of the sample preparation procedure in which no extraction step was employed and errors due to extraction were avoided. This also serves as confirmation that no internal standard was necessary. Several compounds were investigated for use as internal standards, however, and anthranilic acid eluted between PAABA and PAAHA which made it a good candidate for this purpose. The conditions of analysis had to be altered to completely resolve anthranilic acid, and this was accomplished at the expense of analysis time so it was decided to conduct sample measurements without the use of an internal standard.

The limits of detection, taken as the peak height corresponding to twice the baseline noise, was found to be 0.06 mg/L for PABA; 0.12 mg/L for PAHA; 0.11 mg/L for PAABA and 0.24 mg/L for PAAHA.

Recovery. Each compound, at the same concentrations as the aqueous standards, was added to pooled blank urine collected from fasting subjects under the test conditions outlined in the methods section. Recovery was determined by replicate analysis (n = 6) at each

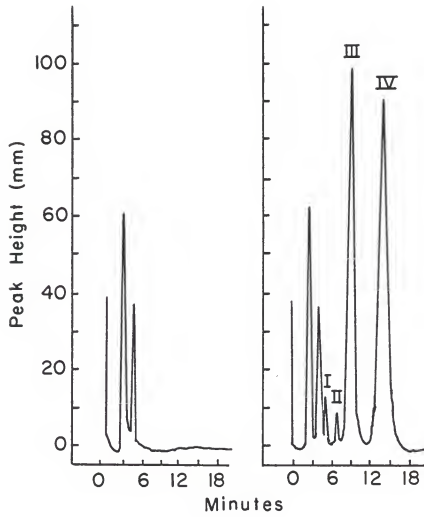


Fig. 3-21. Typical chromatograms under the conditions in table 2-4, of a pooled blank urine (left) and a spiked urine containing 0.5 mg/L p-aminobenzoic acid (I), 0.71 mg/L p-aminohippuric acid (II), 6.52 mg/L p-acetamidobenzoic acid (III), and 12.93 mg/L p-acetamidohippuric acid (IV).

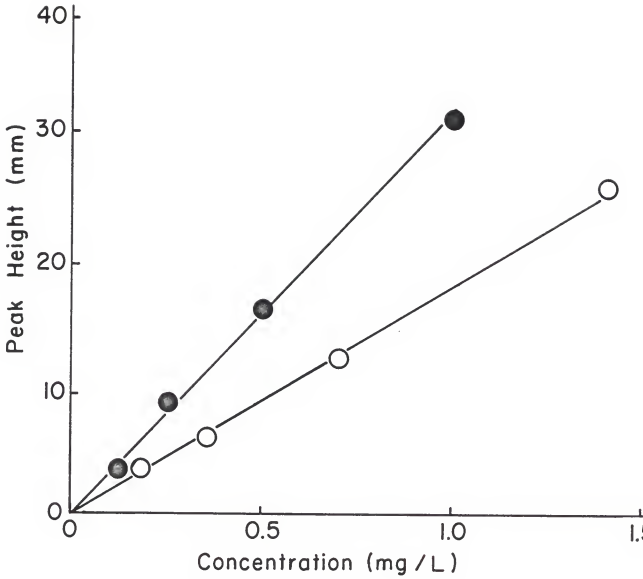


Fig. 3-22. Typical standard curves of peak height versus concentration for p-aminobenzoic acid (closed circles) and p-aminohippuric acid (open circles).

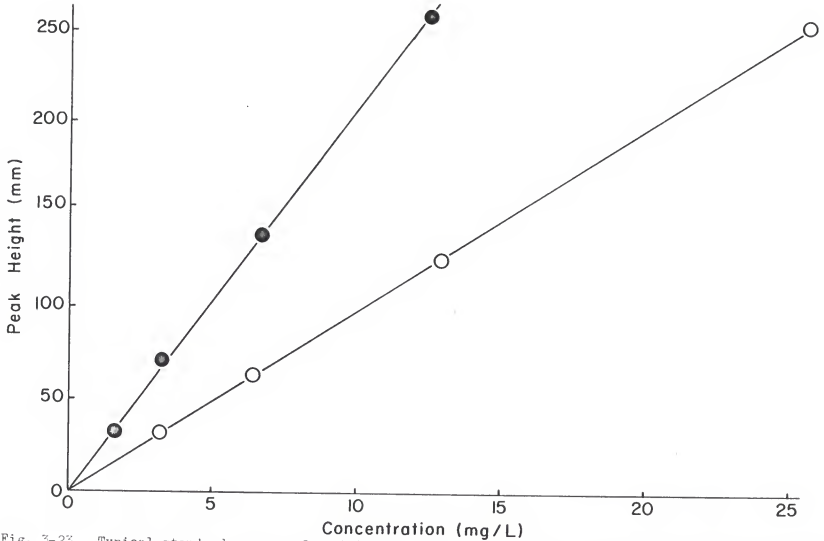


Fig. 3-23. Typical standard curves of peak height versus concentration for p-acetamidobenzoic acid (closed circles) and p-acetamidohippuric acid (closed circles) and p-acetamidohippuric acid (open circles).

concentration. The mean slopes of the peak height versus concentration curves for spiked urine were compared to slopes from curves obtained by direct injection of aqueous standards. The results are summarized in Table 3-9.

Table 3-9. Recovery of PABA and its metabolites by the ion-pair HPLC method

Compound	Concentration range (mg/L)	Mean slope	Standard error	Recovery % (n = 6)
PABA	0.13 - 1.0	30.0	0.23	95
PAHA	0.18 - 1.42	16.7	0.38	98
PAABA	1.63 - 13.04	17.7	0.13	100
PAAHA	3.23 - 25.86	8.4	0.03	100

Precision. The between-day precision was assessed by analyzing the four concentrations of spiked urine pool for 6 days. The coefficient of variation for each compound was less than 10% for all urine concentrations tested (Table 3-10) except for very low concentrations of PABA and PAHA. The within-day run variation in peak height was less than 5% CV at the lowest and highest standard concentrations for all compounds tested (n = 6).

Selectivity. Good selectivity of the method under test conditions was indicated by the fact that no interfering peaks were observed in pooled blank urine (Figure 3-21). The y intercepts of curves constructed from spiked urine were 0.039 ± 0.13 for PABA; -0.66 ± 0.32 for PAHA; 1.78 ± 0.98 for PAAHA and -0.059 ± 0.50 for PAAHA (n = 6)

Table 3-10. Between-day precision of the ion-pair HPLC method

Compound	Mean concentration (mg/L)	C.V. % (n = 6)
PABA	0.98	3.0
	0.48	9.2
	0.25	9.2
	0.13	16.7
PAHA	1.40	3.6
	0.67	2.3
	0.32	5.4
	0.18	14.0
PAABA	12.4	6.5
	6.5	3.8
	3.3	3.2
	1.6	8.3
PAAHA	26.0	2.9
	12.8	1.4
	6.5	1.4
	3.3	5.3

also indicating no significant interferences. Blank urine from non-fasting subjects who were not restricted from drug use, however, did demonstrate many interfering peaks which suggests that test conditions must be rigorously controlled for good selectivity.

Evaluation of Bentiromide Metabolism in Clinical Samples

The development of the ion-pair HPLC method now permits the study of individual metabolite concentration patterns in clinical samples for the purpose of detecting false positive bentiromide test results. In this section, the appropriate urine collection interval will be established through pharmacokinetic studies. The metabolite concentration patterns in normal subjects, pancreatic patients and liver patients will

be determined under the established conditions and evaluated for significant differences. Additionally, various parameters extracted from these metabolite concentration patterns will be evaluated for their effectiveness in the differentiation of false positives due to liver dysfunction.

Pharmacokinetic Studies

It is reasonable to assume that PABA metabolite concentration patterns will vary during the 6 hour collection interval normally used for the bentiromide test. It is also possible for certain metabolites to reach their peak concentrations in urine at different times after a dose of bentiromide has been administered. Thus it is important to investigate this possibility so that potential differences may be optimally determined. The concentration versus time profiles for each metabolite quantified from normal volunteers is presented in Figure 3-24. This experiment was conducted according to the protocol outlined in the methods section of this work and quantitation was achieved by the HPLC method. The concentrations of PABA and PAHA are too low to be useful in the normal subjects tested although it is possible that analytically useful concentrations may be present in hepatic patients. The concentrations of PAABA and PAAHA were high enough to be useful and demonstrated concentration maxima at 2 and 3 hours, respectively. If PAABA and PAAHA concentrations are to be used for the evaluation of PABA metabolism in a single sample, a urine collection interval must be chosen that would best reflect any metabolic lag time. The collection interval of 0-3 hours was initially chosen for single sample analysis because metabolic lag might best be detected prior to the concentration maximum. Also concentrations would be high enough for reliable

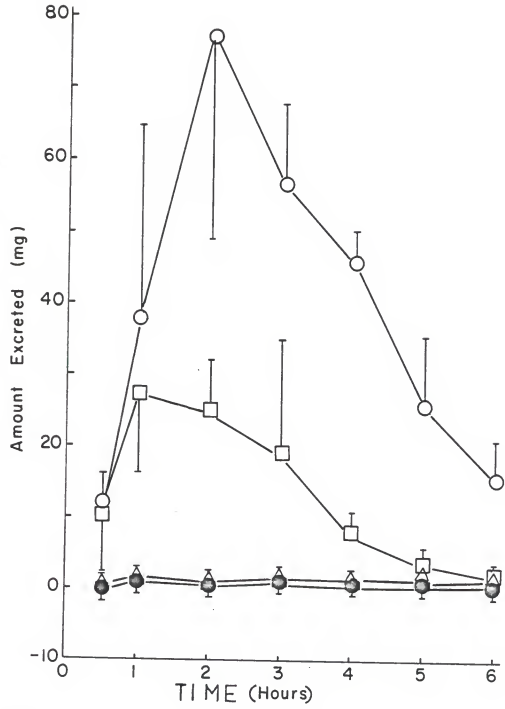


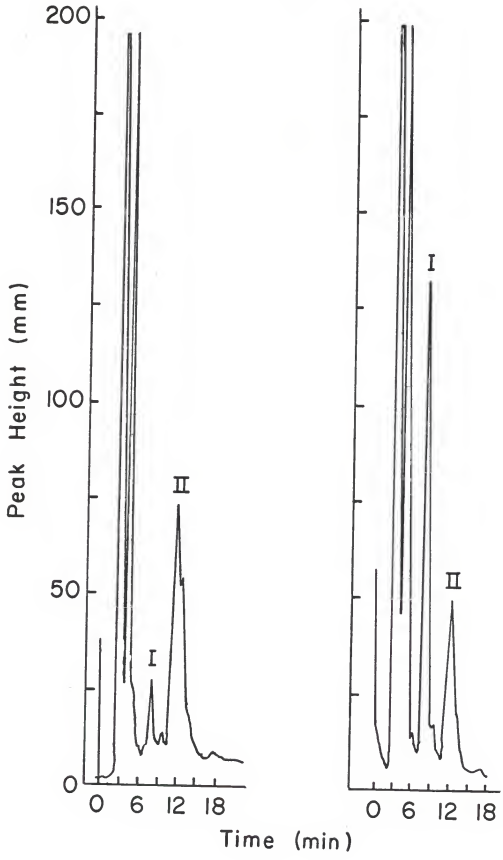
Fig. 3-24. Urinary excretion versus time curves for p-aminobenzoic acid and metabolites (n=5). See figure 3-10 for a definition of symbols and table 2-4 for chromatographic conditions.

quantitation over this interval. The large standard deviation represented in Figure 3-24 is reflective of individual variation in the subjects tested and indicates that metabolite concentrations in liver patients must be widely variant for differentiation.

An attempt was also made in this study to evaluate the pharmacokinetic mechanism of PABA metabolism. Although no meaningful data could be obtained for PABA and PAHA, the data in Figure 3-24 suggests that PAABA is eliminated in approximately first order fashion whereas PAAHA shows zero order elimination. This suggests a metabolic conversion of PAABA to PAAHA and direct renal excretion of PAAHA without further conversion. To prove that this is the predominant mechanism of PAAHA synthesis, however, the rate of formation of PAAHA must be approximately the same as the rate of elimination of PAABA. This study is not possible in urine samples, however, since PAABA is significantly excreted into urine and its elimination must be due, at least partially, to this mechanism.

Another factor which supports the idea that PAABA is further metabolized is that a peak resulting from PAABA glucuronide formation appeared in the chromatograms of samples collected more than 3 hours after administration of bentiromide. The peak was confirmed as PAABA-glucuronide by treatment with glucuronidase which eliminated the extra peak and caused an increase in the PAABA peak height (Figure 3-25). This observation does not support the claim that PAABA is converted to PAAHA, however, and all that can be said about metabolic mechanisms is that PAABA appears to be further conjugated whereas PAAHA appears not to be.

Fig. 3-25. Typical chromatograms under the conditions in table 2-4 of a urine sample collected at the 3-6 hour interval from a subject undergoing the bentiromide test. The chromatogram on the right is from the urine sample after treatment with glucuronidase and the one on the right was untreated. The presence of p-acetamidobenzoyl glucuronide is indicated by the disappearance of the shoulder on the p-acetamidohippuric acid peak (II) and the increase in peak height of the p-acetanidohippuric acid peak (I) after treatment with glucuronidase.


200
150
100
50
0
Peak Height (mm)
I
II
0 6 12 18
I
II
0 6 12 18
Time (min)

Analysis of Clinical Samples

The PAABA-glucuronide peak was not resolved from the PAAHA peak so it was decided to modify the sample preparation procedure of the HPLC method to include a glucuronidase treatment step. The optimum time interval for incubation of samples with glucuronidase was determined by monitoring metabolite peak heights versus time of incubation. The results of this study are presented in Figure 3-26 and show an increase in PAABA peak height accompanied by a decrease in peak height of the unresolved PAAHA-PAABA-glucuronide peaks. Figure 3-26 also shows an increase in PAHA peak height that suggests formation of PAHA glucuronide. The optimum incubation time was chosen as 30 min because that was the minimum time for stabilization of all analyte peak heights. The specificity of the glucuronidase enzyme with respect to analyte species was also validated by this study since no changes in peak heights were observed after 30 minutes of incubation with an excess of enzyme.

All clinical samples were analyzed by the modified procedure so that an accurate assessment of both acetylation and glycine conjugation of PABA could be made. The amount of each analyte excreted over the initial three hour collection period in normal subjects, pancreatic patients and liver patients is presented in Table 3-11. The extremely low amounts of PABA and PAHA excreted and the broad range of values within each population made these results useless for the differentiation of metabolite synthesis. This was proven by statistical evaluation in which no differences were found by comparing population means even at the 50% confidence level. The results from PABA analysis were evaluated by a non-parametric procedure (163) since the

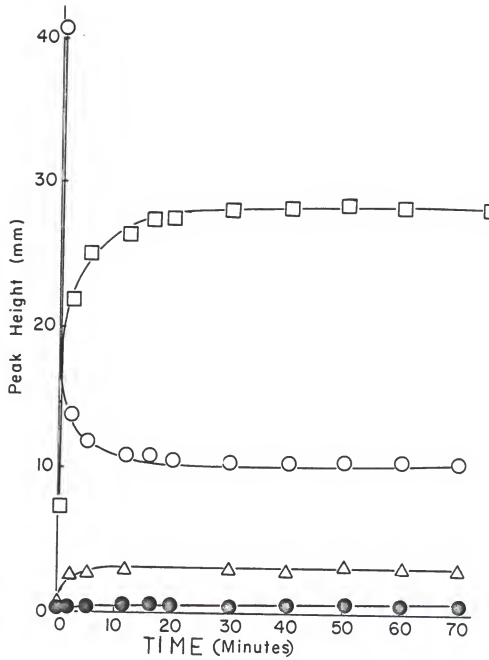


Fig. 3-26. Plot of peak height versus time of treatment with glucuronidase for p-aminobenzoic acid and its metabolites. The chromatographic conditions are listed in table 2-4 and the symbols are the same as for figure 3-10.

Table 3-11. Amount of PABA and its metabolites excreted in clinical samples over a 0-3 hour collection period

Subject population	n	PABA (mg)		PAHA (mg)		PAABA (mg)		PAAHA (mg)	
		mean	range	mean	range	mean	SD	mean	SD
normal	10	0.26	0-1.37	2.41	0.32-5.36	65.8	44.3	36.6	27.8
pancreatic	10	0.46	0-2.17	2.38	1.28-6.30	28.0	16.1	13.9	10.7
liver	5	0.58	0-1.81	1.03	0.30-1.73	64.1	47.3	21.0	17.5

distribution could not be assumed normal because of skewness by results of zero. The results for PAHA, however, were normally distributed and evaluated by the parametric t statistic (149).

The results from PAABA and PAAHA analysis showed promise for metabolite differentiation and suggest that there may be a larger amount of PAABA excreted in liver patients than in either normal subjects or pancreatic patients. However, the large standard deviations observed rendered these differences insignificant even at the 50% confidence level and an alternate strategy was necessary to demonstrate possible differences. Low total recovery of metabolites is expected in pancreatic patients and some liver patients and this would tend to mask individual metabolite differences if only one metabolite was evaluated. A ratio technique was employed, therefore, to correct for variations in total recovery. Since relatively high values for PAABA excretion in liver patients was suspected, the ratio technique used, expressed the ratio of the amount of PAABA excreted to the total amount of PAABA + PAAHA. The mean ratios for normal, pancreatic and liver patients were 0.622±0.114, 0.692±0.113 and 0.738±0.089, respectively. Again, a comparison of these results by the students t test showed insignificant differences although it did appear that there may be an increase in PAABA relative to the amount in liver patients.

At this point, it was decided to evaluate the situation from a pharmacokinetic point of view to see if the sample collection protocol could be altered to demonstrate a significant difference in PABA metabolism among the three groups. It was observed that significant variations occurred in the time at which PAABA and PAAHA reached their maximum levels in individual subjects during the pharmacokinetic study

presented in Figure 3-24. This adds to the variation found in PAABA and PAAHA excretion for the 0-3 hour collection interval since sample collection was halted near the time of maximum excretion. Also, erratic results were observed for the 0-3 hour interval in a study to determine the formation rate of PAABA and PAAHA (Figure 3-27). In this experiment, a longer collection interval and shorter sampling times were used to provide more experimental points for pharmacokinetic analysis but the erratic excretion levels found between 0-3 hours precluded such an evaluation. The results do suggest, however, that there is a greater variation in PAABA and PAAHA excretion during the first three hours after bentiromide administration. Apparently, a steady-state of physiological hydration of the subject which provided a more consistent elimination rate of PABA metabolites is reached after the 0-3 hour interval. Consequently, it was thought that a collection interval of 3-6 or 0-6 hours might be more appropriate for metabolite evaluation and studies were carried out to determine this. Samples collected between 3 and 6 hours after bentiromide administration were also collected during the clinical study and were quantitated to evaluate their usefulness in metabolite differentiation. The results of this study are presented in Table 3-12 and show that there is slightly less variation in metabolite excretion within each study group as indicated by lower relative standard deviations. Statistical evaluation of individual metabolite excretion again showed no significant differences at the 50% confidence level among subject populations. Amounts of PABA and PAHA excreted were evaluated by non-parametric methods in this case and PAABA and PAAHA were evaluated with the parametric t statistic. The ratio of PAABA to total PAAHA + PAABA in these samples was 0.247±0.046 for normal

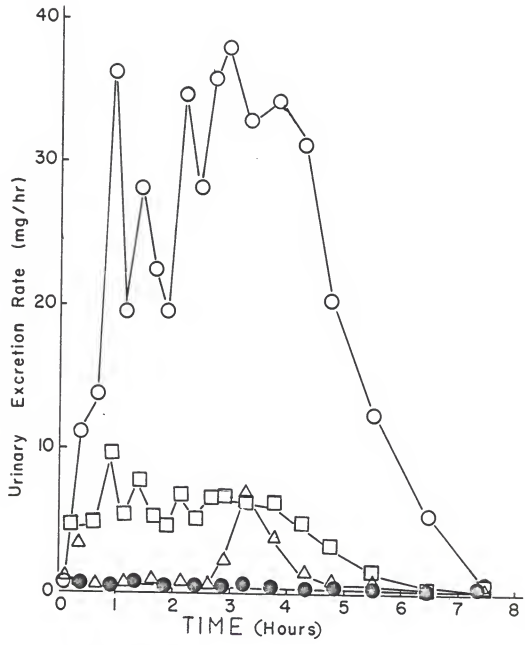


Fig. 3-27. Urinary excretion versus time curve for p-aminobenzoic acid and its metabolites from one subject over an extended time period. See figure 3-10 for a definition of symbols and table 2-4 for chromatographic conditions.

Table 3-12. Amount of PABA and its metabolites excreted in clinical samples over a 3-6 hour collection interval

Subject population	n	PABA (mg)		PAHA (mg)		PAABA (mg)		PAAHA (mg)	
		mean	range	mean	range	mean	SD	mean	SD
normal	10	0.70	0-2.51	0.32	0-1.16	11.8	7.5	35.6	21.3
pancreatic	10	0.38	0-1.39	0.78	0-2.11	26.8	17.8	50.0	18.6
liver	5	0.54	.05-1.45	1.43	0.2-3.36	19.6	10.1	18.1	11.5

subjects, 0.556±0.125 for liver patients and 0.402±0.248 for pancreatic patients. Although there was a significant increase in the ratio between normal subjects and liver patients ($p<0.001$), the difference between both normal subjects and pancreatic patients and between liver and pancreatic patients was not significant. This is because of the large variation observed for pancreatic patients and is probably due to slow PABA recovery resulting from deficient enzymatic cleavage of bentiromide. This would cause an increase in the time of maximum metabolite excretion in these patients and thus cause a shift in the variablity associated with this factor.

A 0-6 hour collection interval was simulated by adding the amounts of each metabolite excreted during the 0-3 collection interval to those from the 3-6 hour interval and the results are presented in Table 3-13. Again, there were no significant differences ($p>0.5$) between individual mean amounts of metabolites excreted, among the study groups. The amounts of PABA and PAABA excreted were still too low for evaluation and highly variant so these two compounds were deleted from further consideration. The ratio technique when applied to the 6 hour data, however, demonstrated significant increases of 0.220 ($p<0.001$) and 0.211 ($p<0.001$) for the liver patient population as compared to the normal and pancreatic populations, respectively. The mean values of the PAABA to total PAABA + PAAHA were 0.427±0.118 for normal subjects, 0.647±0.120 for liver patients and 0.436±0.100 for pancreatic patients. There was no significant difference between the populations of normal and pancreatic subjects ($p>0.5$), however, which indicates that the ratio technique was successful in correcting for variable total recovery.

Table 3-13. Amount of PABA and its metabolites excreted in clinical samples over a 0-6 hour sample collection interval

Subject population	n	PABA (mg)		PAHA (mg)		PAABA (mg)		PAAHA (mg)	
		mean	range	mean	range	mean	SD	mean	SD
normal	10	0.99	0-3.88	2.33	0.32-5.36	81.9	44.7	75.1	29.4
pancreatic	10	0.94	0-2.2	3.29	1.28-8.14	56.3	32.2	66.6	16.4
liver	5	1.12	0.13-3.25	2.46	0.7-4.86	83.8	57.1	38.9	24.5

These studies indicate that liver patients can be distinguished from both normal subjects and pancreatic patients in clinical situations. They also show that only PAABA and PAAHA need be quantitated for this purpose and that a 0-6 hour collection interval with the ratio technique must be used. The diagnostic implications of this study will be discussed in Chapter 4 of this dissertation.

Analysis of Bentiromide Metabolites by Room Temperature Phosphorescence

Throughout the studies on PABA and its metabolites by HPLC, PABA and PAHA were determined to be of little use for the differentiation of patient populations. The major metabolites PAABA and PAAHA, however, are useful for this purpose and will be the focus of the work described in this section. Quantitation of PAABA and PAAHA in clinical samples requires that measurements be made in a mixture of all metabolites unless a separation step is included. Therefore, the room temperature phosphorescence characteristics of all metabolites must be studied to adequately assess the analytical possibilities and provide the most efficient analysis system. The goal in this section will be to differentiate the room temperature phosphorescence of these metabolites from one another and various methods will be investigated for this purpose. If metabolite differentiation can be incorporated into the RTP analytical format, a convenient and rapid method for bentiromide test evaluation which is not subject to false positive tests in liver patients may be provided.

Spectral Studies

The most obvious way to attempt to differentiate the phosphorescence of several compounds in a mixture is by spectral

discrimination. If the RTP emission maxima of PAABA and PAAHA were sufficiently separated from PABA and PAHA and from one another, emitted radiation could be monochromated to filter out emission from all compounds except the one of interest. This possibility was investigated by determining the excitation and emission spectra of each compound individually. The absorption spectrum of each was also determined and the absorptivities in aqueous solution were calculated. The results of these studies are presented in Table 3-14 and the RTP emission spectra on the DE-81 substrate are shown in Figure 3-7. Table 3-14 and Figure 3-7 show that the feasibility of spectral resolution of these four compounds is slight because of the small separations between their emission maxima and the breadth of the emission peaks. The fact that the RTP intensity of PABA was more than 10-fold that of either PAHA or PAABA, and more than 100-fold that of PAAHA, may prove to be useful for individual quantitation of metabolites if a separation is employed. For example, if there were some way to remove PAABA from the sample and not PAAHA, the relative quantities of these metabolites could be determined by difference. This could be accomplished by analyzing the sample by the RTP procedure for total metabolites and then analyzing the separated sample in the same manner. The difference between the two measurements would reflect the relative amounts of PAABA and PAAHA. Since the amounts of PABA and PAHA excreted are negligible, the ratio of the signals from separated and nonseparated samples would be analogous to the ratio shown in the previous section to be significantly different in liver patients than in pancreatic patients, and could potentially be used for diagnostic purposes. To preserve the convenience of the RTP method, however, it would be advantageous to obviate a separation step,

Table 3-14. Some spectral characteristics of PABA and its metabolites

Compound	λ Abs	Molar Absorptivity ($x10^4$), $\frac{abs}{M\ cm}$	RTP λex	RTP λem	RPI*
PABA	270	1.25	294	428	930
PAHA	270	1.47	300	438	64
PAABA	262	1.84	277	425	63
PAAHA	265	1.89	295	437	7

* The relative phosphorescence intensities were from ($1.82x10^{-3}$ M) solutions of each compound.

and other means were investigated to accomplish the analysis without separation.

Selective Heavy Atom Effects

The convenience of the RTP method would not be significantly affected if the selective enhancement of phosphorescence by a heavy atom perturber could be used. Ideally the phosphorescence of either PAABA or PAAHA would be selectively enhanced by a particular heavy atom species making it possible to correlate the ratio of PAABA to PAAHA concentration with the ratios of phosphorescence intensity of the two with and without the heavy atom present. Three heavy atom species were studied for potential selective enhancement effects on PABA and its metabolites and the results are shown in Table 3-15. The iodide ion enhanced the phosphorescence of all test compounds and the degree of enhancement was 3.16-fold for PABA, 4.92-fold for PAHA, 2.90-fold for PAABA and 6.34-fold for PAABA. Tl^+ and Ag^+ enhanced the phosphorescences of the individual analytes to about the same degree with the greater enhancement observed for PAABA. The approximate degree of enhancement for both heavy atom species was 1.75-fold for PAHA, 3.52-fold for PAABA and 3.38-fold for PAAHA. The phosphorescence of PABA, however, was apparently decreased 1.77-fold in the presence of these two heavy atom species.

For the purpose of distinguishing PAABA from PAAHA by selective enhancement in the complex mixture represented by the sample, several factors must be considered. The choice of heavy atom depends on the degree of selective enhancement of phosphorescence required for either PAABA or PAAHA, the enhancement of phosphorescence of PABA and PAHA which would potentially interfere with the measurement and the

Table 3-15. The effect of several heavy atom species on the room temperature phosphorescence intensities of PABA and its metabolites (n=5)

			H_2O		0.1 M KI		0.1 M $TlNO_3$		0.1 M $AdNO_3$	
	λex	λem	RPI mean	SD	RPI mean	SD	RPI mean	SD	RPI mean	SD
PABA (50 mg/L)	294	428	38	1.0	120	8.9	22	1.4	21	1.7
PAHA (354 mg/L)	300	438	57	9.9	300	31.9	107	13.5	115	17.3
PAABA (320 mg/L)	277	425	52	1.8	151	12.8	183	19.4	174	13.7
PAAHA (431 mg/L)	295	437	7.1	0.13	45	2.9	24	1.8	25	1.7

enhancement of phosphorescence of endogeneous components of urine. It appears that the best approach would be to use iodide since the effect on the phosphorescence of PAAHA was more than twice the effect on that of PAABA. However, calculations based on the average concentrations of PAABA and PAAHA found in patients suffering from liver and pancreatic diseases by HPLC, demonstrate that the percent increase in total phosphorescence intensity from all analytes due to PAAHA enhancement would be only 15.1% for the pancreatic disease population and 7.0% for the liver population. Although this reflects the higher relative amount of PAAHA excreted in the pancreatic population, the difference of 8.1% is within the limits of experimental error established for the RTP method. This small difference is due to the relatively low contribution of PAAHA phosphorescence to the total phosphorescence and negates the analytical utility of this approach.

The use of Tl^+ or Ag^+ to selectively enhance PAABA with respect to PAAHA would be a better choice if the fraction of total phosphorescence was the only important factor because PAHA and PAAHA phosphorescences are not enhanced as much as by iodide and the phosphorescence of PABA is decreased. However, the percent increase in total phosphorescence due to selective PAABA phosphorescence enhancement would be only 0.4% higher in the liver patient population than the pancreatic patient population because of the small difference in enhancement between PAABA and PAAHA. Although it was not studied because of the small differences found in these initial experiments, the enhancement of endogeneous urinary components would lower these differences by increasing total phosphorescence and it appears that selective enhancement is not a viable means to differentiate PAABA and PAAHA in clinical samples. The

observations made here are valuable in characterizing the physical aspects of the phosphorescence of these compounds and a discussion of these aspects will be included in Chapter 4.

Lifetime Studies

Time resolution is another potential means of distinguishing between PAABA and PAAHA without a physical separation step. If the phosphorescence lifetime of one of these components is sufficiently longer than those of the other components, phosphorescence measurement can be made after a specified delay time in which the phosphorescences of all other components have vanished and the signal is proportional to only the concentration of the longer lived component. This is an ideal situation, however, and such lifetime differences are not commonly observed for structurally related compounds such as PABA and its metabolites. It may be possible, however, to relate relative concentrations of the compounds of interest through a study of phosphorescence relative intensities and lifetimes with respect to their expected concentrations. The phosphorescence lifetimes of PABA and its metabolites spotted from aqueous solution onto untreated and iodide treated DE-81 filter paper are presented in Table 3-16. These lifetimes were determined from phosphorescence decay curves in which phosphorescence intensity was measured versus time after cutting off the exciting light. The lifetime was calculated by regression analysis of the natural logarithm of phosphorescence intensity versus time curve and the lifetime was taken as the inverse of the slope of this curve. The lifetime determined in this way is the time it takes for the phosphorescence intensity to decrease to a fraction (1/e) of the initial phosphorescence intensity.

Table 3-16. Phosphorescence lifetimes of PABA and its metabolites in the presence and absence of heavy atom

	No heavy atom			1.0 M KI added		
	τ_p(ms)			τ_p(ms)		
	mean	SD	r*	mean	SD	r*
PABA	388.7	0.02	0.996	148.7	0.04	0.998
PAHA	571.2	0.00	1.002	157.9	0.05	0.997
PAABA	438.7	0.03	0.992	109.8	0.09	0.994
PAAHA	916.8	0.03	0.997	82.9	0.11	0.995

* r is the value of the correlation coefficient of the ln RPI versus time curve.

The two possibilities for time discrimination of PAABA and PAAHA are to gate out the phosphorescence of PAABA in the absence of a heavy atom or to gate out the phosphorescence of PAAHA in the presence of a heavy atom, since the lifetime of PAABA is relatively shorter for the former and longer for the latter case. The lifetimes of PAABA and PAAHA are different by a factor of only about 2 in each case, however, and completely gating out the phosphorescence of one analyte would result in low signals from the second. The interference from PABA and PAHA must also be considered in each case since their lifetimes are also similar.

Consider first the temporal resolution of PAAHA from PAABA in the absence of a heavy atom at a gate time of 1.8 sec. The phosphorescence intensities of PABA, PAHA, PAABA and PAAHA would be reduced by a factor of $(1/e)^{4.6}$, $(1/e)^{3.2}$, $(1/e)^{4.1}$ and $(1/e)^{2.0}$, respectively, at that gate time. Again, if the resulting RTP intensities are based on a calculation from RTP standard curves involving the RTP intensities from mean concentrations of analytes found in liver and pancreatic patients, the total phosphorescence intensity would be reduced 94.9% in the pancreatic patient population and 96.0% in the liver patient population. The higher signal calculated for the pancreatic patients reflects the higher relative concentration of PAAHA although the difference in populations is only 1.1% and well within the range of error for the method. Shortening of the gate time would only decrease the difference between populations and a longer gate time would result in total phosphorescence intensities that were too low for reliable analytical measurements.

Similar calculations were carried out for the temporal resolution of PAABA from PAAHA in the presence of a heavy atom and at a gate time of 0.2 sec. In this case, the mean total phosphorescence intensity of

PABA and its metabolites would be decreased 77.8% for pancreatic patients and 77.1% for liver patients which again reflects higher relative PAABA excretion in the liver patient population. The difference of 0.7% is not useful analytically and an alternate strategy was necessary to incorporate metabolite differentiation into the RTP format.

Differential Hydrolysis of Metabolites

It has been shown in studies represented by Figures 3.4 and 3.5 that acid hydrolysis of PAABA to PABA is 95% complete after 15 minutes whereas acid hydrolysis of PAAHA to PABA is only 4% complete. Alkaline hydrolysis, on the other hand, is complete for both compounds after 1 hour. This difference in hydrolysis characteristics of PAABA and PAAHA can be used to differentiate these metabolites by carrying out separate acid and base hydrolysis procedures on aliquots of the same sample. The differences between phosphorescence intensities of these hydrolysis mixtures should correlate with the differences in relative PAABA and PAAHA concentrations found in subject populations. This correlation is made possible by the fact that liberated PABA demonstrates much higher phosphorescence intensity than its metabolites and the total contribution to the phosphorescence signal from unconverted metabolites in the acid hydrolysis mixture would be small. Thus the ratio of total phosphorescence intensity measured from PABA in the acid hydrolysis mixture to that of the alkaline hydrolysis mixture should approximate the ratio of PAABA concentration to the total PAABA plus PAAHA concentration measured by HPLC.

The degree of interference from metabolites of PABA in the acid hydrolysis procedure after 15 minutes at 100°C can be estimated by

calculating the relative phosphorescence intensities that would result from the concentrations of metabolites that were determined by HPLC in that mixture. These concentrations were 10 mg/L of PABA, 286 mg/L of PAHA, 0 mg/L of PAABA and 27 mg/L of PAAHA which resulted from acid hydrolysis for 15 minutes of 451 mg/L of PAAHA. The total phosphorescence contribution from these concentrations is only 17.6% of the phosphorescence that would have been produced if all of the PAAHA had been converted. This would thus, provide a difference between acid and base hydrolysis of 80.4% (allowing for incomplete PABA recovery) in the amount of apparent PABA liberated from PAAHA. These calculations were based on the use of the DE-81 substrate without heavy atom treatment because all heavy atom species studied enhanced the phosphorescence of PAHA and PAAHA more than that of PABA.

These calculations indicate that the method has the best potential for discrimination of PAABA and PAAHA of all the methods studied and a study of clinical samples was carried out to evaluate the method. The alkaline hydrolysis was carried out on clinical samples in the usual manner and the acid hydrolysis was stopped after 15 minutes by neutralization of the mixture with 2 M NaOH containing 0.01 M KH_2PO_4. The results of the determinations in these hydrolysis mixtures of clinical samples is presented in Table 3-17. The relative phosphorescence intensities from acid and base hydrolyses appears to reflect the increased amount of PAABA excreted by liver patients since this ratio is higher than that obtained from both the normal and pancreatic patient populations. All of the ratios are higher, however, and the difference is not as great as for ratios calculated from HPLC data. This was expected since the phosphorescence from unconverted metabolites of PABA

Table 3-17. Amount of apparent PABA measured in clinical samples by room temperature phosphorimetry following acidic and alkaline hydrolysis

Subject population	n	PABA (mg) (alkaline hydrolysis) mean	SD	PABA (mg) (acid hydrolysis) mean	SD	acid to base ratio mean	SD
normal	10	110.9	52.0	71.5	28.0	0.644	0.180
pancreatic	10	85.8	35.3	53.8	15.6	0.627	0.143
liver	5	90.9	59.6	72.2	45.5	0.794	0.153

in the acid hydrolysis mixture would interfere with the quantitation of PAABA by adding to the observed signal. Statistical analysis of the data shows that the differences among all populations for acid versus base hydrolysis are not significant even at the 50% confidence level. The differences between ratios are somewhat more significant, however, and the liver population demonstrated ratios that were different from normal and pancreatic patient populations at 90% and 80% confidence levels, respectively. The difference between normal and pancreatic populations was again insignificant at the 50% confidence level.

CHAPTER 4
DISCUSSION

Potential sources of error that are associated with the bentiromide test as it is presently administered have been treated separately in this work. The problem of lack of analytical specificity was improved by the development of the RTP method for PABA analysis. Physiological non-specificity with regard to liver patients was also treated by the development of the liquid chromatographic method for PABA and its metabolites and the evaluation of metabolite excretion patterns in clinical subjects by this method. An RTP method was also developed for the differential analysis of PABA metabolites and evaluated with clinical samples.

In this section, a detailed discussion of the analytical merits of each method will be presented. Also, the variables involved in analytical development will be addressed from a mechanistic point of view and the diagnostic value of the methods will be evaluated in detail. During the course of this study, information relating to the physical aspects of room temperature phosphorescence of PABA and its metabolites was obtained and will be discussed here.

Room Temperature Phosphorimetry of p-Aminobenzoic Acid

The choice of substrate for PABA analysis was based on drying times, the dependence of RTP signals on pH, relative detection limits, and commercial availability. The DE-81 substrate was superior to the DTPA impregnated S&S 903 substrate in all categories except for

drying characteristics. Currently accepted RTP theory suggests that rigid binding of the analyte to substrate material is one of the primary factors contributing to high phosphorescence intensity (94). So far, hydrogen bonding is believed to be the main mechanism responsible for the rigidity factor in filter paper substrates (44,94). This hydrogen bonding occurs between analyte molecules and hydroxyl groups present on cellulose in the paper substrate. The relatively high phosphorescence intensities observed on the DE-81 substrate are probably due to a combined effect of hydrogen bonding and ionic bonding between the ionic exchange groups on the DE-81 substrate and analyte molecules. This combined effect results in a more rigid binding of the analyte molecule to the substrate than for substrates which do not possess ion exchange groups and thus, results in higher observed phosphorescence.

The DTPA impregnated S&S 903 substrate demonstrates superior phosphorescence at room temperature because of a different mechanism. It was initially thought that the addition of DTPA would remove trace amounts of transition metals in filter paper substrates (164) that might contribute to phosphorescence background. Treatment with DTPA, however, gave no improvement in blank signals, but did significantly improve the analyte signal. The effect on the analyte signal was attributed to interaction of the analyte with DTPA (a strong hydrogen bonding agent) and to filling of the gaps in porous filter paper substrates. This resulted in analyte molecules being trapped in the DTPA-cellulose matrix (142) and causing higher phosphorescence intensities as a result of stronger binding to the substrate and a higher surface concentration of analyte molecules because of less penetration of the analyte into the substrate. Another possible explanation of the improved analyte signal

when DTPA is added to the S&S 903 substrate is that metal ions whose interaction with the analyte results in decreased phosphorescence are chelated. In this way, the analyte would be protected from interaction with these metal ions and higher phosphorescence intensities would result.

The faster drying time observed for the DTPA treated S&S 903 substrate could also be due to the higher surface concentration of analyte molecules. The flow of dry nitrogen is directed onto the surface of substrate sample discs, therefore, the water removing nitrogen would act faster on molecules concentrated on the surface of the substrate than for those which have penetrated into the depths of the paper. The DE-81 filter paper is also considerably denser than the DTPA treated S&S 903 filter paper and water removal from the denser substrate is expected to take a longer time. The rise times of phosphorescence were shortened and the plateau times were increased for both substrates when 50% ethanol was used as the spotting solvent. The increase in phosphorescence rise time observed when ethanol was added was probably due to increased volatility of the ethanol-water mixture as compared to water alone. Plateau times may be related to the strength of analyte binding to the substrate, since decreases in phosphorescence following the plateau are probably due to desorption of analyte molecules. The DTPA treated S&S 903 substrate demonstrated longer plateau times and slower fall rates which suggests that its interaction with analyte molecules is probably stronger than the ionic interaction of the analyte with charged groups on DE-81. This is also suggested by the observation that DTPA treated S&S 903 demonstrated greater phosphorescence signals than DE-81 without the heavy atom added. The

fact that the addition of 50% ethanol increased the plateau times on both substrates is probably related to the lower viscosity of this medium which causes greater spreading of analyte molecules along with greater penetration into the substrate. A corresponding decrease in total phosphorescence intensities with 50% ethanol added to the application mixture was not observed, however, which does not support this claim. The decrease in fall rate for DTPA treated S&S 903 observed when ethanol was present in the application mixture also does not support this claim and an exact explanation of the mechanistic effect of ethanol is not possible with the data presented here.

The environment of the DE-81 substrate is basic (151) and the diethylaminoethyl group may be expected to have some buffer capacity. This explains the extended pH range in which the DE-81 substrate provided relatively stable phosphorescence intensities. The range is extended to a much greater extent in the basic region for DE-81 as compared to the DTPA impregnated S&S 903 substrate. This is because of the basic nature of the DE-81 substrate and its ability to buffer solutions applied to it in the basic pH range. The range of stable phosphorescence in the acidic region is also slightly extended for the DE-81 substrate, suggesting that the substrate has the ability to neutralize acidic spotting solutions to some extent. The phosphorescence intensity versus pH curve for the DTPA treated S&S 903 substrate was stable over a smaller range of pH because of an apparent lack of buffer capacity of the substrate.

The decrease in phosphorescence signal in strongly acidic solutions (below pH 2.5) is probably due to protonation of either the carboxyl group or the amino group on PABA which prevents strong binding to the

substrate. The pH at which this decrease occurs for both substrates supports protonation of the amino group as the primary cause of the decrease and the fact that the quaternary ammonion group on DE-81 would not be expected to bind strongly with a positively charged analyte also supports this. Von Wandruska and Hurtubies (66) proposed that the decrease observed at high pH in the room temperature phosphorescence of PABA on a sodium acetate substrate was due to precipitation of NaOH upon evaporation, but did not offer an explanation why this would result in lower phosphorescence intensity. A possible explanation for the loss of phosphorescence intensity in the presence of precipitated NaOH is the hygroscopic nature of NaOH crystals. The water attracted to the substrate by NaOH crystals would result in lower RTP intensities (65). This study suggests that there may be a competition for substrate binding sites because the DE-81 substrate has a much greater anion binding capacity than the DTPA treated S&S 903 and also showed stronger RTP in the basic region. Another possible contribution to the decrease in RTP intensities observed in the basic region for DTPA treated S&S 903 is the possible loss in chelation of metal ions, which interact with the analyte, by DTPA in that pH range.

The higher RTP intensities observed from the DE-81 substrate in the presence of iodide is easily explained in terms of heavy atom binding to the substrate. The anionic exchange filter paper, DE-81, binds anions such as iodide making them more accessible to analyte ions which are also bound to the substrate.

The increased iodide heavy atom effect for DE-81 as compared to DTPA treated S&S 903 was present in both blank and analyte studies which suggests that the observed effect is a property of the substrate and not

the analyte-heavy atom interaction on the substrate. The positively charged heavy atom species tested on the DE-81 substrate gave lower room temperature phosphorescence intensities than untreated DE-81 which suggests either competition of the analyte with heavy atom species for substrate binding sites or an increased rate of radiationless deactivation processes caused by the spin-orbital coupling effect of the heavy atom (165).

The performance of the RTP method in clinical samples was observed to have certain advantages and disadvantages when compared to other analytical methods. The range of linearity for the RTP system is superior to that of both colorimetric methods (21,42) but not as wide as that for HPLC with electrochemical detection (154). The RTP method is less precise than both colorimetric and HPLC procedures, but analytical variability is overshadowed by physiological variability which is encountered in the bentiromide test and the contribution to method error due to analytical imprecision is small for the RTP method.

Another advantage to RTP analysis by the proposed method is complete conversion of all PABA metabolites back to the parent compound. The acid hydrolysis, which is commonly used in colorimetric procedures, does not completely convert PAAHA to PABA but rather converts it to the intermediate PAHA. Fortuitously, this metabolite reacts in both colorimetric procedures to form a chromophore similar to that of the PABA-chromogen complex. However, any differences in reactivity or absorptivity of these complexes combined with the observed individual variability in PABA metabolism could lead to erratic results. Additionally, colorimetric methods are non-specific and urinary aromatic amine contamination would cause false positive

results. Extreme care must be taken to avoid these interferences and interfering drugs must be discontinued at least 3 days prior to the test. This might necessitate the interruption of critical drug therapy which is not possible, in some cases. Electrochemically detected HPLC is seemingly not subject to such interferences although complete drug interference studies have not been carried out with the HPLC method.

The RTP method is relatively specific with regard to the compounds tested except for procaine which does not pose a therapeutic problem if discontinued. Also, metabolite recovery studies and sample blank analysis indicate that endogeneous urinary components and hydrolysis by-products do not interfere with the method. The detection limit is more than adequate for clinical analysis of urine samples and provides the potential for PABA analysis in blood.

The RTP method is more rapid and convenient than either HPLC or colorimetric procedures and readily applicable to routine analysis. Although some of the equipment necessary is not commercially available, slight modifications of commercially available front surface attachments could be used with slight modification (141). The diagnostic performance of the bentiromide test with RTP detection of recovered PABA can be considered to be essentially equivalent to that for colorimetric methods if care is taken to avoid drug interferences. This is validated by the good correlation of the two methods under these conditions and the diagnostic sensitivity and specificity for the method would be roughly equivalent to 72% and 95%, respectively.

Variables Involved in the Chromatographic Technique

Brief explanations regarding the observations that were made during development of the HPLC procedure for PABA and its metabolites have

already been presented in Chapter 3 of this dissertation to rationalize the choices made. Therefore, a discussion of these factors here will be limited to an overview of the factors involved and the analytical merits of the system.

It was suggested in the results section of this work that methanol was a less selective solvent (produced smaller k' values) for adjusting the capacity factors of PAABA and PAAHA as compared to acetonitrile. The proposed explanation is that the amides (PAABA and PAAHA) are better hydrogen bonding species (154) than the amines (PABA and PAHA) and would, therefore, interact more strongly with a proton donating and accepting solvent (155) such as methanol. The more strongly the solvent interacts with specified sample components, the more preferentially it will dissolve the sample compound in question, thus decreasing the retention provided by the stationary phase. There are actually three major interactions between solute and solvent molecules that must be considered in determining the primary mechanism of retention; dispersion, dipole and hydrogen bonding forces.

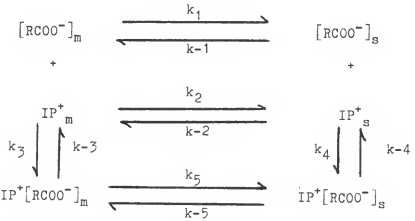
Dispersion interactions which result from induced dipolar moments are stronger for compounds having highly polarizable electrons and are related to the refractive indices of solvent and solute molecules. The refractive indices of acetonitrile and methanol are 1.341 and 1.326 (156), respectively, which suggests a very small difference in the strength of dispersion interactions between the two solvents. This indicates that the differences in retention observed for analyte molecules in methanol and acetonitrile are probably not due to dispersion interactions.

Dipole interactions are observed to occur between molecules having permanent dipole moments. These interactions occur with solvent and sample molecules that have relatively polar functional groups and the interaction is stronger with functional groups that are strongly electron donating or withdrawing. The dipole moments of the hydroxyl group on methanol and the nitrile group on acetonitrile are 1.7 and 3.5 debyes, respectively (110). This relatively large difference in dipole moments between the main functional groups of the two solvents studied indicates that there is a significant difference in dipole interactions with solute molecules. The interaction should be stronger for acetonitrile, however, and this is not consistent with the observations made in this work. This suggests that differences in dipole interactions were not the primary reason for the preferential interaction of solvent molecules with PAABA and PAAHA.

Hydrogen bonding interactions were chosen as the likely mechanism to explain greater interactions between methanol and the acetylated analyte molecules because of the greater hydrogen bonding capacity of the amide group (155). Amides represented by PAABA and PAAHA interact more strongly with hydrogen bonding solvents by virtue of their ability to form multiple hydrogen bonds. Although the amino substituents on PABA and PAHA are more strongly basic and thus better hydrogen bond acceptors, the amido substituents can form hydrogen bonds at both the nitrogen and carboxyl oxygen sites. This creates a combined effect for the amido compounds that is stronger than the individual effect corresponding to the amino derivatives.

The concept of ion-pair equilibria which was introduced in Chapter 1 and referred to in Chapter 3 requires further discussion here so that

the results observed may be described in more detailed terms. The following set of simultaneous equilibria is necessary to adequately describe the entire system of ion-pair chromatography:



(30)

where $RCOO^-$ represents negatively charged analyte molecules and IP^+ represents a positively charged pairing-ion molecule. The subscripts m and s signify mobile and stationary phases, respectively, and the foreward and reverse rate constants are represented by k with positive or negative subscript numbers indicating forward and reverse rates, respectively. The simultaneous equilibria described in (30) shows a distribution of free analyte ions, pairing-ions and ion pairs between the mobile and stationary phases at rates corresponding to k_1, k_2 and k_5. The diagram also shows that ion-pairs are formed at different rates in the mobile and stationary phases.

It is very difficult to determine the exact cause for observations involving ion-pairing agents with only capacity factor data, because the observed effects could be due to a number of contributing factors. For example, it was proposed in the results section of this work that the deviation from linearity observed for the pairing agent hexadecyltrimethylammonium bromide, which did not belong to the homologous series

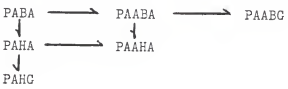
represented by the other pairing agents, was due to either alteration of the stationary phase because of pairing-ion binding or an increased ion-pair formation constant because of better accessibility of the hexadecyltrimethylammonium charged head. It is not possible to distinguish the cause of nonlinearity with capacity factor data and each possible cause could in turn be due to several factors. Alteration of the stationary phase could be due to increased hydrophobicity attributed to binding of the pairing ion (k_2) or an apparent ion exchange effect represented by the rates k_1 and k_4. The higher potential accessibility of the charged head on hexadecyltrimethylammonium bromide could also be due to either a higher ion-pair formation rate in the mobile phase (k_3) or a higher ion pair formation rate in the stationary phase (k_4). It is also obvious from the equations in (30) that changing any of the rates will in turn affect the other rates and further complicate definitive evaluation.

In the study of anionic ion-pairing agents, it was observed that the capacity factors of PAABA and PAAHA increased at pH 2.5 with increasing pairing-ion size. The pKa's of the amido groups are considerably lower than 2.5 (see Figure 3-12), however, which suggests that retention by the pairing-ion must involve alteration of the stationary phase because ion-pair formation with these compounds would not be expected at that pH. The overall effect on retention by anionic pairing agents was much greater for PAHA and PABA which indicates ion-pair formation must be considered because these analytes are ionized at the pH studied. These two observations are good evidence for the combined effect which was proposed in the results section, but the exact contributions cannot be determined.

The HPLC method for PABA and its metabolites that was developed is only slightly less precise than the method of Ito (153) for analysis of PABA in which an internal standard was used. The HPLC method was proven useful for differentiation of metabolite excretion in liver and pancreatic patients if the test conditions are carefully controlled, which reflects good accuracy of the method in clinical samples. With modifications, the method can be applied to the quantitation of PABA and its metabolites in blood to enable in depth pharmacokinetic studies that may reveal a better understanding of PABA metabolism.

Detection or Falsely Positive Bentiromide Test Results by Metabolite Analysis

Although the primary pathway of PABA metabolism cannot be definitively obtained by analysis of metabolites in urine samples, there was some information gained in this study which can be treated in qualitative terms. The pathways that could have resulted in the metabolites that were found are shown in (31).



(31)

Para-acetamidobenzoyl glucuronide and p-aminohippuryl glucuronide are represented in (2) by PAABG and PAHG, respectively. These two compounds were shown to exist in urine samples of subjects undergoing the bentiromide test by recovery of PAHA and PAABA after treatment with glucuronidase. The observation that PAABG was only found in urine samples collected at least 2 hours after bentiromide administration and after high levels of PAABA were reached, indicates that PAABG is formed

from PAABA and not through a glucuronide conjugated intermediate of PABA. All three groups of subjects studied demonstrated a much greater ratio of PAABA to PAAHA in earlier (0-3 hr collection interval) samples than in later samples (3-6 hr collection interval) which indicates that conversion of PAABA to PAAHA is the primary pathway for PAAHA synthesis rather than PAHA to PAAHA. This is also substantiated by the fact that no appreciable amounts of PAHA were found in urine samples and that the amount of PAHG was also a small fraction of the total amount of metabolites. The amount of PAAHA produced in normal subjects is approximately equal to the amount of PAABG as shown by HPLC analysis of PABA and its metabolites, in samples before and after treatment with glucuronidase. This suggests that the rates of formation of PAABG and PAAHA from PAABA are similar.

All of these interpretations should be regarded as only qualitative descriptions of the most likely metabolic pathway since good quantitative pharmacokinetic data could not be obtained from the urine samples. It can be said with certainty that the major metabolites of PABA in all groups of clinical subjects studied are PAABA, PAAHA and PAABG. These results agree with studies done in dogs (27) and guinea pigs (28) where two dimensional thin layer chromatography and liquid-liquid extraction were used as the separation techniques. Following treatment with glucuronidase, the major metabolites present for the differentiation of metabolism in liver patients are PAABA and PAAHA. It was shown in the results section of this work that the most significant parameter for the evaluation of liver function by PABA metabolism was the ratio of PAABA to total PAABA + PAAHA in glucuronidase treated urine samples collected over a 6 hour collection interval. The results from

HPLC analysis of PABA metabolites were shown to be more reliable for this purpose than analysis by differential hydrolysis of PAABA and PAAHA followed by RTP measurement.

The 95% confidence interval for mean ratios from pancreatic and liver population is 0.363-0.508 and 0.480-0.813, respectively, as analyzed by HPLC. Although the means of these populations were shown to be significantly different at $p<0.01$, there is some overlap of the two 95% confidence intervals which is more closely related to the clinical utility of the method. The 95% confidence interval can be used as a value for the normal range within each population that would include 95% of that population. Defined in those terms, there is a 19.0% overlap of the pancreatic population with that of the liver population and an 8.4% overlap of the liver population with the pancreatic population. These Figures can be related to diagnostic sensitivity and specificity because the number of false positives (pancreatic patients diagnosed as having liver dysfunction) is related to the degree of overlap between the pancreatic patient confidence interval and the liver patient confidence interval. Similarly, the degree of overlap of the liver patient population with that of the pancreatic patient population is related to the number of liver patients that would be evaluated as not having liver dysfunction (false negatives). The value for diagnostic sensitivity can then be predicted to be 84.5% and the diagnostic specificity 68.1% which reflects the percent of positive tests that were accurately tested as positive and the percent of negative tests that were accurately tested as negative, respectively. If the cut-off point for liver dysfunction is taken to be the lower limit of the 95% confidence interval for the mean ratio of PAABA to PAABA + PAAHA, only 2

out of the 10 pancreatic patients tested in the clinical study would have had ratios above the cut-off point and been tested as positive for liver dysfunction. This yields an observed diagnostic specificity of 66% which agrees well with the predicted value. Among the 5 liver patients tested, none had ratios that were below the cut-off point so the observed diagnostic sensitivity was 100%. This does not agree well with the predicted value although better agreement would probably be obtained with a larger sample of the liver patient population.

The diagnostic value of PAADA to PAABA + PAAHA ratios determined by differential hydrolysis with detection by RTP would not be expected to be as good because of the observed interferences from unconverted metabolites in the acid hydrolysis procedure. The 95% confidence interval for liver and pancreatic patient populations in this case were 0.625-0.962 and 0.519-0.735, respectively. The degree of confidence interval overlap for the liver population was 32.6% and that for the pancreatic population was 50.9%. The predicted values for diagnostic sensitivity and selectivity with the RTP method were 50.8% and 32.5%, respectively. The observed diagnostic sensitivity in the clinical samples was 42.8% and the diagnostic selectivity was also 42.8%. It is obvious from these calculations that the diagnostic value of the RTP method for metabolite analysis is inferior to that of the HPLC method. Although the RTP method is more selective analytically with regard to endogeneous components of urine, the poor diagnostic value of the results makes the method less useful for the defined purpose.

Physical Aspects of the Room Temperature Phosphorescence of Para Aminobenzoic Acid and Its Metabolites

Various studies related to the phosphorescence characteristics of PABA and its metabolites were carried out in an effort to resolve their RTP signals for analytical purposes. Although these attempts were unsuccessful because of inadequate differences among the wavelengths of maximum emission, heavy atom effects and lifetimes of the individual analytes, the data allow a discussion of the physical aspects of these observations which will be presented here. Before a detailed discussion of the observed RTP characteristics can be presented, it is necessary to briefly describe origins of the lowest excited triplet state.

The phosphorescent properties of a molecule are determined by the nature of population of the lowest excited triplet state. The rates of intersystem crossing (ISC) from singlet (S_1) to triplet (T_1) states generally increase as the energy difference between these states decrease (166). Singlet and triplet states from $\pi\pi^*$ electronic configurations that are derived from transitions involving lone pair electrons on substituent groups lie lower in energy than $\pi\pi^*$ states of the unsubstituted compound. This will result in the substituted molecule having a smaller degree of singlet-triplet energy splitting, if the lowest excited triplet $\pi\pi^*$ state is less shifted to lower energy than the lowest excited singlet $\pi\pi^*$ state. The smaller degree of energy splitting in the substituted molecule would result in an enhanced rate of ISC and contribute to enhanced phosphorescence efficiency.

Benzoic acid did not show any phosphorescence at room temperature whereas the amino derivative of benzoic acid (PABA) showed very strong phosphorescence. This observation could be due to the S_1 and T_1 states

in PABA originating from electronic configurations involving lone pair electrons on the amino nitrogen that are closer in energy than those of benzoic acid. The belief that S_1 is lower in PABA is substantiated by a 34 nm red shift in the absorption spectrum of PABA relative to benzoic acid.

The metabolic conjugation of the substituent groups on PABA results in delocalization of the electron donating and accepting effects attributed to these substituents. This is one possible explanation for the lower phosphorescence intensities observed for metabolites of PABA. The phosphorescence intensities of both the acetyl derivative (PAABA) and the glycine derivative (PAHA) are about 14-fold lower than that of PABA. When PABA is both acetylated and glycine conjugated (PAAHA) the phosphorescence at room temperature is decreased approximately 133-fold. The phosphorescence emission maximum of PAHA and PAAHA were red-shifted 10 and 9 nm, respectively (relative to PABA) whereas the PAABA conjugate is blue-shifted only 3 nm. The corresponding excitation maxima were red-shifted 6 nm for PAHA and 1 nm for PAAHA and blue-shifted 17 nm for PAABA. The resulting energy splitting of the S_1 and T_1 states are 34 nm for PABA, 38 nm for PAHA, 48 nm for PAABA and 42 nm for PAAHA. The greater energy splitting of S_1 and T_1 for all metabolites of PABA relative to the parent compound is a possible explanation for the decrease in phosphorescence efficiency of these compounds. This is a very simplified explanation, however, because both the radiative and nonradiative processes that deactivate the triplet state depend on not only S_1 and T_1 but also on the nature of the intermediate triplet states which affect the deactivation process. Therefore, alterations in the degree of energy splitting between higher

triplet states and T_1, which may have been caused by the delocalizing effect of metabolic conjugation on PABA substituents, could have resulted in lower phosphorescence intensities.

Lifetimes and Heavy Atom Effects

The presence of heavy atoms of high atomic number in the vicinity of a phosphorescent molecule enhances its probability of phosphorescence by increasing the rate of intersystem crossing and the rate of phosphorescence from the triplet state. The heavy atom effect can affect both radiative and nonradiative singlet-triplet transitions and is purely electronic in nature (167). The quantum yield of phosphorescence (ϕ_p) is defined by

$$\phi_p = \phi_{ST} \frac{k_p}{k_p + k_{TS}} \qquad (32)$$

where k_p is the rate constant of phosphorescence, k_{TS} is the sum of all unmolecular or pseudo first order rate constants for radiationless deactivation of the triplet state and ϕ_{ST} is the efficiency of singlet-triplet crossing from the lowest excited singlet state. Equation (32) shows that phosphorescence quantum yields can be decreased by the heavy atom effect if k_{TS} is increased to a great extent relative to ϕ_{ST} and k_p. This is a possible explanation for the decrease in phosphorescence intensity observed for PABA in the presence of Tl^+ and Ag^+. However, the phosphorescences of all of the metabolites of PABA were enhanced by Tl^+ and Ag^+ which suggests that in these compounds k_p and/or ϕ_{ST} increase faster than k_{TS} as a result of enhanced spin-orbital coupling.

The relative effects of the iodide heavy atom on phosphorescence intensities and lifetimes of PABA and its metabolites demonstrate a

pattern which can be related to the type of metabolite conjugation. In order to discuss this pattern, the lifetime of the triplet state (τ_p) must be defined in terms of intersystem crossing and triplet state deactivation rates as follows.

$$\tau_p = \frac{\phi_{ST}}{k_p + k_{TS}} \tag{33}$$

In qualitative terms, if τ_p is increased ϕ_{ST} increases faster than $k_p + k_{TS}$. Such is the case with all PABA metabolites relative to PABA and the glycine conjugates demonstrate the greatest increase in τ_p. Correspondingly if ϕ_p is decreased, as is also the case with the metabolites of PABA, ϕ_{ST} k_p decrease faster than $k_p + k_{TS}$ (equation 32). Therefore, k_p decreases faster than k_{TS} for all metabolite molecules relative to PABA since ϕ_{ST} in the metabolites is increased relative to $k_p + k_{TS}$. This suggests that the increase in phosphorescence lifetimes observed for the metabolites of PABA can be attributed more to a decrease in k_p rather than a decrease in k_{TS}. Also, the phosphorescence quantum yields of PABA metabolites were decreased by decreases in k_p since $\phi_{ST}/k_p + k_{TS}$ is increased for all compounds. The portion of phosphorescence intensity decrease which was due to decreases in k_p is greater for PAHA and PAAHA than for PAABA because of the longer lifetimes of the former. The amount of phosphorescence intensity decrease relative to PABA was about the same for PAHA and PAABA so the phosphorescence intensity decrease due to increases in k_{TS} and or decreases in ϕ_{ST} must have been greater for the acetylated derivative.

This can be shown in quantitative terms by dividing equation 32 by equation 33 to yield the following expression.

$$\frac{\phi_p}{\tau_p} = k_p \qquad (34)$$

Although the absolute quantum yield of room temperature phosphorescence was not determined because of the lack of a quantum yield standard, the relative k_p values can be calculated for each compound of interest by determining phosphorescence intensities at concentrations that would yield the same absorbances and substituting these values for quantum yields. The values of k_p calculated in this manner were 0.93 for PABA, 0.12 for PAHA, 0.18 for PAABA and 1.2×10^{-3} for PAAHA.

The relative contributions of ϕ_{ST} and or k_{TS} can be estimated by solving equation (33) for ϕ_{ST} and substituting values for k_p and τ_p. The equation for each compound can then be normalized so that it is in the form:

$$X\,\phi_{ST} - Y\,k_{TS} = 1 \qquad (35)$$

where X and Y are coefficients derived from the data for k_p and τ_p which are inversely related to the relative magnitudes of ϕ_{ST} and k_{TS}. Although the values for ϕ_{ST} and k_{TS} can not be separated by this technique, the sum of contributions from $\phi_{ST} + k_{TS}$ can be ordered from highest to lowest showing relative effects. The order of contributions from $\phi_{ST} + k_{TS}$ for the compounds of interest were found to be PABA > PAABA > PAHA > PAAHA.

The values calculated for k_p show that the glycine conjugated derivative of PABA loses phosphorescence from a decrease in k_p upon

conjugation more so than does PAABA. The order of $\phi_{ST} + k_{TS}$ indicates that PAABA loses phosphorescence because of a decrease in ϕ_{ST} and or an increase in k_{ST} more so than does PAHA. The PAAHA metabolite shows the greatest decreases in phosphorescence by both mechanisms which represents the combined effect of altering both substituents on PABA.

In the presence of the iodide heavy atom, the relative values of k_p were calculated to be 7.3 for PABA, 2.2 for PAHA, 2.0 for PAABA and 0.32 for PAAHA. The interesting aspect here is that the relative magnitudes of the k_p values for PAHA and PAABA are reversed upon addition of the heavy atom. The order of the degree of contributions from $\phi_{ST} + k_{TS}$ for PABA and its metabolites is PABA > PAHA > PAABA > PAAHA. Again, the relative contributions to a loss of phosphorescence intensity by decreases in ϕ_{ST} and or increases in k_{TS} were reversed for PAHA and PAABA. There were no changes observed in the energy splitting of the lowest excited triplet state and the ground singlet state in the presence of the heavy atom.

There are several statements that can be made with certainty about the relative phosphorescence characteristics of PABA and its metabolites. The rate constant of phosphorescence is decreased by both acetylation and glycination of PABA. The combined decreases in rates of singlet-triplet intersystem crossing and nonradiative deactivation of the triplet state which contribute to a loss of phosphorescence intensity are enhanced by both acetylation and glycination. The combined effect of acetylation and glycination decreases phosphorescence intensity to a degree that is greater than the sum of the individual effects. There is also a difference in the mechanism of phosphorescence reduction between

glycination and acetylation which is reversed upon treatment with the iodide heavy atom.

Exactly how these observations relate to the origin of electronic states and the delocalizing effects of metabolic conjugation can only be speculated. It is reasonable to assume, however, that the effects observed for acetylation of the amino group are related to interaction of the electron withdrawing acetyl group with the $\pi\pi^*$ state originating from transitions involving lone pair electrons on the amino group. Also, the effects observed for glycination must be due to a direct effect on the $\pi\pi^*$ state originating from transitions involving the transfer of electron density from the aromatic ring to the carboxyl group. The $\pi\pi^*$ states that arise from charge transfer from the aromatic ring to the carboxyl group should be viewed as involving charge transfer from the lone pair of the donor (amino group) to the π orbital associated with the acceptor (carboxyl group). It appears from the data that glycination of the carboxyl group results in an increase in its electron withdrawing ability, thus decreasing the energy of transition. This was observed as red shifts in both the excitation and emission phosphorescence maxima for PAHA. The blue shifts associated with acetylation of the amino group are probably due to a decreased electron donating effect since the acetyl group is more strongly electron withdrawing than the hydrogen atom.

In all cases, these shifts in $\pi\pi^*$ state energies resulted in an increased energy gap between S_1 and T_1 relative to PABA. The observation that the decrease in phosphorescence of PAABA was due more to decreases in ϕ_{ST} and/or increases in k_{TS} rather than decreases in k_p (relative to PAHA) is consistent with the observed spectral shifts since the energy gap between S_1 and T_1 is larger for PAABA.

The Effect of Substrate Binding

The effect of analyte binding to the substrate must also be considered as a source of the observed differences in phosphorescence intensities. The amino and carboxyl groups on PABA can interact with the DE-81 substrate by hydrogen bonding of the amino groups to hydroxyl groups on the substrate and by ionic interactions of the carboxylate group with the alkyl ammonium ion of the substrate. A similar mechanism for the binding of PABA to sodium acetate was described by von Wandruszka and Hurtubise (66), which showed that this type of parallel orientation of analyte molecules to the solid surface maximized binding.

The lower phosphorescence intensities observed for glycinated and acetylated derivatives of PABA could be due to weaker interactions with the substrate because of the delocalization of charge on the substituent groups. The carboxamido group on PAHA may be less conjugated with the aromatic ring than the carboxylate group on PABA and less able to withdraw electron density from the ring. This would result in an apparent decrease in the negative charge of the carboxamido group on PAHA relative to the carboxylate group of PABA and may result in a weaker interaction. The acetylated amino group on PAABA could also cause a change in the charge density relative to PABA. This may result in weaker binding because the amido group on PAABA is less basic than the amino group of PABA. The acetylated conjugate, however, also has a carboxyl oxygen atom that could contribute to hydrogen bonding with hydroxyl groups, thus enhancing binding.

Another possible mechanism for weaker binding of the glycine conjugates is that of intramolecular hydrogen bonding between amino and carboxyl groups. This would compete with intermolecular hydrogen bonding

to the substrate and would result in weaker bonding. The relative binding of PABA and its metabolites cannot be determined without further study and the mechanisms described here should be considered hypothetical.

Conclusions

This study on the analytical aspects of the bentiromide test has yielded data which indicate that room temperature phosphorimetry is a superior analytical method to those in current use. Analysis of bentiromide metabolites by ion-pair high performance liquid chromatography has shown that ratios of certain metabolite concentrations can be used to differentiate false positive bentiromide test results due to liver dysfunction. These metabolite concentration ratios can also be determined by room temperature phosphorimetry with differential hydrolysis although this method is inferior to the chromatographic method in terms of diagnostic value. The appropriate urine collection interval for analyses of metabolite ratios was determined to be six hours and the major metabolites of bentiromide were identified as p-acetamidobenzoic acid, p-acedamindohippuric acid and p-aceamidobenzoyl glucuronide.

In addition to analytical and clinical studies, the physical aspects of the phosphorescence of bentiromide metabolites were studied. The rate constant of phosphorescence at room temperature is decreased to a different degree by both acetylation and glycine conjugation of p-aminobenzoic acid. The singlet-triplet intersystem crossing efficiency is decreased and/or the rate constant for non-radiative deactivation of the excited triplet state is increased by both acetylation and glycination of p-aminobenzoic acid. The combined effects of acetylation and glycination decrease phosphorescence at room temperature to a degree that is greater than the sum of the additive individual effects.

APPENDIX I
INTERFERING SUBSTANCES

acetaminophen
acetophenetidin
aspirin
atropine
benzocaine
chloramphenicol
chlorothiazide
indomethacin
lidocaine
metaclopramide
neomycin
oral pancreatic enzyme supplements
phenobarbital
procaine
procainamide
sulfonamides
thiazide diuretics

APPENDIX II
CLINICAL EVALUATION

1. History - to include biographic data, history, and review of systems.

2. Physical Exam - to include height, weight and a thorough systematic examination.

3. Vital Signs - to include supine blood pressure, pulse, respiration and body temperature (oral).

4. Clinical laboratory - to include the following:

   Hematology - hematocrit, red blood cell count, total white blood cell count and differential white blood cell count.
   Serum chemistry - BUN, SGOT, alkaline phosphatase and creatinine.
   Urinalysis - specific gravity and urine qualitative chemistry.

APPENDIX III
EXCLUSION CRITERIA

1. History of having received any investigational drug during the four weeks prior to entrance into study.

2. History of hepatic, pancreatic or renal disease.

3. History of alcoholism or excessive alcoholic consumption.

4. History of malabsorption.

5. Treatment with any drugs listed in Appendix 1 at time of initial evaluation.

6. Presence of any significant pretreatment clinical laboratory findings.

7. Requirement for any concomitant medication listed in Appendix 1 during the period of the study.

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BIOGRAPHICAL SKETCH

H. Thomas Karnes was born in Cincinnati, Ohio, on September 27, 1952. He was graduated from Peoria High School in Peoria, Illinois, in 1970. He attended Illinois Central College in East Peoria, where he received an Associate of Arts degree majoring in medical technology in May, 1973. In August, 1974, he began study at Illinois State University where he received a Bachelor of Science degree in chemistry in May, 1977. He entered the Graduate School at the University of Florida in September, 1977, where he served as a graduate assistant in the Department of Chemistry and subsequently in the Department of Pathology where he received a Master of Science degree majoring in clinical chemistry in December, 1980. From January, 1981, to August, 1982, he was employed by the Departments of Clinical Chemistry and Pharmacy Practice at the J. Hillis Miller Health Center with the responsibilities of developing an analytical toxicology laboratory and analytical support for pediatric pharmacokinetics, respectively. While pursuing his graduate education, he has published nine articles and one book chapter in the area of drug analysis.

H. Thomas Karnes is married to Susan D. Hansen and has one child. He is a licensed medical technologist and a member of the American Pharmaceutical Association, the American Association of Pharmaceutical Sciences, the American Association for Clinical Chemistry and the American Chemical Society. Upon receiving his doctorate, he will join

the faculty at the Medical College of Virginia in Richmond as an assistant professor.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

S. G. Schulman, Chairman
Professor of Pharmacy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephen H. Curry
Professor of Pharmacy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Assistant Professor of Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1984

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